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GERMLINE MINISATELLITE MUTATIONS IN
HERRING GULLS: INDUCED MUTATIONS AT COLONIES
SITUATED NEAR STEEL MILLS

By

CAROLE LYN YAUK

A Thesis
Submitted to the School of Graduate Studies
in Partial Fulfillment of the Requirements
for the Degree of
Doctor of Philosophy

McMaster University

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GERMLINE MINISATELLITE MUTATIONS
IN HERRING GULLS
DOCTOR OF PHILOSOPHY (1998)   MCMASTER UNIVERSITY
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TITLE:   GERMLINE MINISATELLITE MUTATIONS IN HERRING GULLS: INDUCED MUTATIONS AT COLONIES SITUATED NEAR STEEL MILLS

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Abstract

The study of induced heritable mutations occurring in nature is difficult as mutations are extremely rare and large sample sizes are needed to measure statistical differences between populations.

Minisatellites are tandemly repeated units of DNA, 10 to 100 bp in length, predominantly found in non-coding regions of the genome. With multi-locus DNA fingerprinting, several loci are examined simultaneously increasing the sampling efficiency. Minisatellites have the highest mutation rate detected in the genome to date, making them ideal targets for mutational screening.

I examined minisatellite mutation rates in herring gulls (*Larus argentatus*) colonizing areas near steel mills, urban centers, and rural areas of the Great Lakes, to assess the application of minisatellite DNA mutation rates as biomarkers for induced germline mutations. I showed that gulls inhabiting contaminated environments near steel mills inherit significantly more mutations than gulls from rural locations. I found a negative correlation between proximity to industrial coking ovens and minisatellite mutations; the mutation rate increased almost 3 fold from rural to the most heavily impacted site.

My data were not consistent with either of the alternative hypotheses put forth to explain differences in minisatellite mutation rates: (1) genetic differences in the
populations and (2) differences in the age of parents sampled (i.e., older parents may be more susceptible to mutations). I suggest that minisatellite mutations are induced by chemical contaminants in the industrial environment, and that minisatellite DNA provides a sensitive biomarker for induced germline mutations in urban industrial environments.

I investigated the levels of DNA adducts in herring gulls from our primary steel industry site, Hamilton, Ontario, and from one rural location, in order to clarify exposure. The levels and patterns of DNA adducts in erythrocytes of gulls from these two locations were identical, suggesting that DNA adducts in gull erythrocytes are not appropriate biomarkers of exposure.
Preface

There are seven chapters in this thesis. The first chapter is a General Introduction to the project and the seventh is a synthesis and statement of future recommendations. The remaining chapters have been written as manuscripts for publication in peer-reviewed journals. At the present time, chapters 2 and 3 have been published (reprinted with permission), chapter 5 is in preparation for submission to Science, chapter 4 is in preparation for submission to Journal of Great Lakes Research, and chapter 6 is in preparation to submit to Mutagenesis. Information about the title, authors and individual contributions to each of the chapters is as follows:

Chapter 2: “Multi-locus DNA fingerprinting reveals high rate of heritable genetic mutation in herring gulls nesting in an industrialized urban site.”

Authors: C.L. Yauk and J.S. Quinn
Contribution: Field work and lab work was performed with the assistance of numerous field and laboratory assistants. The statistical analysis and the writing were conducted by the candidate. The research was conducted under the guidance of J.S.Q.

Chapter 3: “Monitoring for induced heritable mutations in natural populations: Application of minisatellite DNA screening.”

Author: C.L. Yauk
Contribution: This review was written by the candidate under the guidance of J.S.Q.
Chapter 4: “Genetic structure among Great Lakes and Eastern Canadian herring gull (Larus argentatus) populations.”

Authors: C.L. Yauk and J.S. Quinn
Contribution: Field work, DNA extractions, DNA fingerprinting, statistical analyses and the manuscript writing were performed by the candidate with the assistance of laboratory and field technicians under the supervision of J.S.Q.

Chapter 5: “Induced minisatellite germline mutations in herring gulls (Larus argentatus) colonizing locations near steel industries.”

Authors: C.L. Yauk, G.A. Fox, B.E. McCarry and J.S. Quinn
Contribution: Field work and laboratory work were performed by the candidate with the assistance of laboratory and field technicians. The statistical analyses were performed by Y. Dubrova of Leicester, U.K. The writing was performed by the candidate. The work was performed under the guidance of J.S.Q.

Chapter 6: “DNA adducts in herring gulls (Larus argentatus) from a rural and industrial location using $^{32}$P-postlabelling.”

Authors: C.L. Yauk, B.E. McCarry, D.W. Bryant and J.S. Quinn
Contribution: Field work and laboratory work were performed by the candidate with the assistance of laboratory and field technicians. The statistical analyses and the writing was performed by the candidate. All work was performed under the guidance of J.S.Q, B.E.M and D.W.B.
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CHAPTER 1

GENERAL INTRODUCTION
1.1 Genotoxic Contaminants in the Environment

Increased industrialization has resulted in the widespread distribution of toxic chemicals. Genotoxic chemicals, or mutagens, possess the ability to induce DNA mutations. Despite our knowledge of the release of these chemicals into our environment, we have little understanding of the genetic impacts of genotoxic chemicals on populations because their evaluation in nature is very difficult, as mutations are extremely rare. Efficient metabolism and DNA repair mechanisms result in low mutation rates, generally rendering statistical comparisons between populations insignificant. Furthermore, chemicals are not emitted as single agents or simple binary mixtures. Thousands of chemicals are introduced into our environment daily and the resultant mixtures may vary spatially and temporally. Therefore, the complex nature of exposure to chemicals makes causal links between exposure and effect extremely difficult to determine.

Predictions on the potential manifestation of genotoxic effects in the field based on chronic or acute exposures to mutagens in the laboratory are problematic for several reasons: (1) inbred strains of laboratory animals are not representative of natural populations, (2) laboratory studies usually expose animals to artificially elevated concentrations of chemicals, (3) the nature of exposure to complex mixtures in the field can not be reproduced in the laboratory (i.e., complex mixtures of contaminants as well as spatial and temporal variations) and (4) the consequences of a lifetime of exposure to chemical mixtures can not be reproduced in the laboratory. Therefore, the complex nature of exposure to genotoxins in the environment may result in unexpected mutagenic response in the field. Some wild animal species may be exposed to higher concentrations of genotoxic chemicals than humans (e.g., fish exposed to contaminated sediments).
These organisms may serve as sentinels to quantify mutagenic effects or potential genotoxic risk or to measure the effectiveness of remedial actions.

1.2 Standard Methods for Screening for Induced Mutations

The mutagenicity of complex mixtures can be assayed through fractionation of components of the environmental mixtures, followed by testing these components with standard laboratory bioassays (Marvin et al., 1993; DeMarini, 1994). This technique, called bioassay-directed fractionation, has increased our understanding of the mutagenic properties of mixtures including: (1) the chemicals that account for the majority of the mutagenicity in mixtures, (2) the mutagenic potency of components of mixtures, as well as the overall potency of mixtures from various sources, (3) the types of mutations induced and (4) nucleotide and position specificity within genes for induced mutation (i.e., mutational spectra) after exposure to specific chemicals. Bioassay-directed fractionation has allowed us to identify key mutagens in environmental mixtures, and target groups of highly exposed organisms for further study.

Investigation of the genotoxic effects of chemicals in natural populations of organisms has been investigated primarily in somatic cells. Somatic mutations are important at the level of the individual (i.e., development of diseases like cancer) but these mutations are not heritable, and therefore do not directly translate into effects at the population level. The types of DNA damage commonly measured in field studies include: covalent DNA adducts, single and double strand breaks in DNA, frequencies of sister chromatid exchanges, micronuclei formation, chromosome aberrations and DNA sequence
mutations. In the following sections I will review the techniques currently used and their application to germline mutation screening.

1.2.1 DNA adducts and strand breaks

Chemical exposure can result in direct modification of DNA to produce DNA nucleotide adducts (a covalent bonding of the chemical to the nucleotide). In most cases, metabolic enzymes convert the substance into a reactive intermediate species which reacts with DNA bases to form DNA adducts. Other common types of lesions that are measured include DNA double and single strand breaks. Both of these endpoints have been used to investigate DNA damage in somatic cells, and they provide information that may link induced mutation and environmental genotoxin exposure. Measures in germ cells have not been undertaken at this time, but would be valuable indicators of potential germ cell mutation.

The response to genotoxic chemical exposure in the environment can be measured by estimating the number of DNA single and double strand breaks (SSB/DSB) in circulating nucleated blood cells or in target tissues. DSBs and SSBs in DNA can result from a variety of sources including direct exposure to exogenous mutagenic agents or indirect SSBs resulting from excision of adducts induced by the contaminants. DSBs are thought to result when SSBs occur on opposite strands of DNA in close proximity (Ward, 1988) and are the primary lesions leading to chromosomal aberrations (reviewed in Phillips and Morgan, 1993). The frequency of SSBs provides an estimate of the steady state of damage and repair and is taken as an early warning signal for chromosome damage.
Levels of SSBs and DSBs are elevated in various populations of organisms exposed to chemical contaminants and radionuclides (e.g., Calderón-Garcidueñas et al., 1996; Sugg et al., 1995; Everaarts et al., 1994; Shugart and Theodorakis, 1994; Pandrangi et al., 1995). Monitoring SSBs and DSBs is important because they are good indicators of genetic stress that may translate into permanent genetic damage at the DNA sequence and chromosome level. However, the repair of a SSB is usually error free, and has no direct effect on mutation frequency. Therefore, strand breaks provide only an indirect measure of the mutagenic potential of chemical mixtures and generally are evaluated in somatic cells so they reveal little about the contribution of mutagens to induced heritable mutations.

The quantification of chemical adducts in DNA is an excellent determinant of biologically effective dose of an environmental genotoxin (reviewed in Nestmann et al., 1996). Adducts may be bulky chemicals (e.g., polycyclic aromatic hydrocarbons) that present blocks in the replication process (Brown and Romano, 1991). Such alterations in chemical structure can result in changes in the DNA base sequence. Smaller lesions (e.g., oxidative damage) may promote base mispairing (Shibutani et al., 1991) and other types of damage (Collins et al., 1996). The frequency of DNA adducts is a measure of the distribution and metabolism of a chemical genotoxin and its interaction with the target DNA of specific cell types. The number of adducts bound to the DNA in target tissues of an organism is an effective measure of the integrated dose of exposure to DNA-binding mutagens, and for chronic exposures, reflects the steady state condition between DNA adduct formation and repair.
Chemical exposures, either as mixtures or as single compounds, can produce significantly elevated numbers of DNA adducts in both occupational and environmental settings. For example, humans exposed to contaminants in their occupation (Perera et al., 1994; Hemminki, 1995; Ovrebo et al., 1992) or exposed to high levels of environmental pollution (Perera, 1992), can show an increased frequency of DNA adducts. Among wildlife, pigeons exposed to urban air pollution (Schilderman et al., 1997) and fish populations exposed to contaminated sediments (Dunn et al., 1987; Malins and Gunselman, 1994; Stein et al., 1994) show increased levels of DNA adducts. Care must be taken in the interpretation of DNA adduct data because there may be species-specific, naturally occurring DNA modifications that may complicate data analysis (Kurulec and Gupta, 1993). Additionally, the metabolic system of the study organism should be considered, as some animals may not metabolize certain compounds. The presence of DNA adducts is a useful tool to monitor potential risk for malignancies and mutation (Nestmann et al., 1996). However, like strand breaks, the number of DNA adducts is measured in somatic tissue and is an indirect measure of mutation, as DNA adducts may be repaired. Therefore, the quantity of DNA adducts may not reflect the direct damage of DNA to germ cells.

Although DNA adducts and DNA strand breaks are indirect measures of mutagenicity, the measurement of these lesions is important in demonstrating exposure to mutagens capable of binding to DNA, or capable of inducing strand breakage, and therefore potentially able to induce mutations. An understanding of the number and types of adducts and strand breaks found in different tissues from organisms exposed to contaminants can provide an understanding of the distribution of contaminants to different
cell types, including germ cells. The role of DNA adducts and strand breaks in mutagenesis is still unclear. Correlating increased DNA adducts and strand breaks in germ cells with transmitted germline mutations will strengthen our understanding of the link between induced mutation and environmental genotoxin exposure.

1.2.2. Chromosome damage

Chromosomal alterations may be numerical (e.g., aneuploidy resulting from nondisjunction at meiosis) or structural. An assessment of structural mutation in chromosomes should include frequencies of chromosome and chromatid aberrations, micronuclei induction and sister chromatid exchanges. The type of aberration induced can be influenced by the stage of cell cycle for any particular cell at the time of contaminant exposure (Natarajan, 1993). A powerful technique, fluorescent in situ hybridization (FISH), allows the identification of specific regions of cellular DNA or RNA within chromosome preparations, fixed cells or tissue sections (Swiger and Tucker, 1996). The use of chromosome-specific and region-specific DNA probes permits rapid scoring of large numbers of cells resulting in increased sensitivity. This technique has greatly enhanced the ability to detect agents capable of inducing somatic and germline chromosomal alterations.

Certain types of chromosomal damage have been directly linked to adverse health effects. For example, it has been estimated that 15% of all recognized pregnancies (greater than 5 weeks gestation) in humans are lost before term and as many as 50% of these have detectable chromosome aberrations (Hook, 1983; Shelby et al., 1993). It has been assumed that embryos containing chromosomal aberrations are frequently spontaneously aborted (Shelby et al., 1993) thus rendering the evaluation of the frequency of inherited mutations on the basis of chromosomal aberrations very difficult.

Micronuclei (MN) are formed by lagging fragments of chromosomes or whole
chromosomes at anaphase of cell division (Natarajan et al., 1996). A number of studies on fish exposed to contaminated sediment and water in situ have shown higher levels of micronuclei in exposed populations (reviewed in Al-Sabti and Metcalfe, 1995). It has also been suggested that induction of micronuclei in fish may be used as a biomarker for the potential exposure of humans to genotoxins in their drinking water. However, presently there are a number of unresolved problems in the application of micronuclei induction frequency as biomarkers of in situ genotoxic exposure. The primary problem results from high inter-individual variability that can result from age, sex, season, viral disease or nutritional differences (reviewed in Hughes and Hebert, 1991).

Sister chromatid exchanges (SCEs) are thought to occur as a result of DNA recombinational events that result in the exchange of genetic material between two chromatids of a single chromosome at apparently homologous sites. Increased SCEs correlate to increased levels of DNA strand breakage resulting from exposure to chemicals that may form DNA adducts, cause depurination or depyrimidation, or otherwise interfere with DNA repair and metabolism (Natarajan et al., 1996). Since SCEs involve the reciprocal exchange of identical chromosomal segments, it is an indirect measure of mutation and it is not clear how these events may affect fitness (Hebert and Luiker, 1996).

The determination of the number of MN or SCE's is accomplished by the examination of circulating lymphocytes, or other somatic target cells, and therefore does not reflect heritable genetic damage. Measurement of chromosome aberrations in germ cells (typically sperm) of exposed organisms can indicate the potential for transmission of mutation related to contaminant exposure, but these damaged chromosomes are not necessarily transmitted (Shelby, 1994). Therefore, this is not an endpoint that measures heritable DNA damage in the offspring of individual organisms exposed in the field. Finally, chromosomal aberrations are very sensitive biological end points reflecting the gross effects of chemicals on the genome, in contrast to point mutations, which reflects the effect more directly.
1.2.3. DNA sequence mutations

With the development of numerous molecular biology techniques including the polymerase chain reaction (PCR), much progress has been made in the analysis of induced DNA sequence mutations. Mutations measured at the DNA sequence level include: base-pair substitutions (transitions, transversions), frameshifts resulting from small deletions or insertions, or large scale genomic rearrangements like deletions, insertions, duplications, translocations and inversions. The primary focus of studies investigating DNA sequence mutations has been in humans on genes like the hypoxanthine phosphoribosyl transferase gene, or on cancer-related genes like p53. These studies investigate the generation of somatic mutations following chemical exposures. Determination of heritable mutations at coding loci is extremely problematic as the majority of mutations are deleterious. Therefore, the cost and sample sizes needed to investigate germ cell mutations in coding regions has prevented the undertaking of such programs at this time. However, with increased automation, development of rapid and non-invasive sampling and screening methods, and increased knowledge of mutational spectra (especially knowledge of mutation susceptible DNA sequences, i.e., 'hotspots'), questions on induced coding region DNA sequence mutations should be clarified in the near future.

1.2.4 Mutational 'hotspots'

It has become evident that the mutation rate throughout the genome is heterogeneous. Variation in mutation rate throughout the genome may result from differences in protein-DNA complexes, transcriptional status, coding versus non-coding regional repair, functional DNA versus DNA of unknown or negligible function and the intrinsic nature of the DNA sequence (Boehr, 1994; MacLeod, 1995). The regions of the genome that show the highest rate of mutation are minisatellite DNA sequences (Jeffreys et al., 1985). Selectively screening areas of the genome known to have elevated rates of
mutation increases the chance of detecting statistically significant differences between populations.

1.3 Minisatellite DNA

The most hypervariable regions in the genome of organisms are composed of tandemly repeated arrays of nucleotides, 10 to 100 bp in length, referred to as minisatellite DNA. The elevated mutation rate at minisatellite regions results in high variability of the number of tandem repeats at each locus in natural populations of organisms. The highest minisatellite mutation rate found to date is $6 \times 10^{-2}$ mutations per gamete per generation compared to $1.0 \times 10^{-9}$ base substitutions per year at unique sequence coding DNA loci in humans (Vergnaud et al., 1991; Jeffreys et al., 1985). The presence of these polymorphic regions has resulted in the development of the powerful tool of multi-locus DNA fingerprinting. DNA fingerprinting, as the name suggests, reveals individual-specific banding patterns. The power in the technique lies not only in the hypervariability of the regions screened, but also in its ability to screen multiple loci simultaneously, increasing the sampling efficiency to assess allelic differences in individuals.

DNA fingerprinting has been extensively used in forensic sciences for individual identification (Jeffreys et al., 1985b, 1991), for parentage analysis (Jeffreys et al., 1991) and also in population genetics (Lynch, 1991; Burke et al., 1991). Recently, minisatellite markers have been applied to the field of genetic toxicology. As minisatellite mutations arise predominantly in the germline, the rates of minisatellite mutations in offspring sampled in natural populations have been quantified in order to determine the impact of
mutagens in the environment on germ cell mutagenesis (Yauk and Quinn, 1996, this thesis, Dubrova et al., 1996; Dubrova et al., 1997). Minisatellite markers may provide the only system currently available to screen for germline mutations in the field (Dubrova et al., 1996; Dubrova et al., 1997). A review of minisatellite DNA, and its application to the field of genetic toxicology, is presented in chapter 3.

1.4 Alternative hypotheses for increased rates of minisatellite mutations

Differences in minisatellite mutation rates may be attributed to three causes: (1) differences in the genetic make-up of the populations, (2) differences in the ages of parents sampled (i.e., older parents may be more susceptible to mutations) and (3) presence of genotoxic chemicals in the environment. Therefore, in order to thoroughly investigate genotoxic exposure on minisatellite mutation rates, genetic differences in the population or age differences in the parents need to be carefully examined.

The presence of population differences at the minisatellite loci scored is an important consideration in this study. Not all minisatellite loci undergo the same rate of mutation. For example, Jeffreys et al. (1988) examined five different human minisatellite loci and found that the mutation rates obtained varied substantially between loci. The minisatellites λMS1 and CEB1 are the most unstable found to date with mutation rates of 5.2 and 6% per gamete respectively (Jeffreys et al., 1988; Vergnaud et al., 1991). In the context of the present study, such subsets of loci scored only in gulls from steel sites would result in an over-estimation of induced mutation rates at these colonies. For example, if gull populations from rural sites have a subset of minisatellite alleles from highly mutable loci that are shorter than at steel sites (and therefore scored less frequently
because short alleles may be run off the DNA fingerprinting gels), the resultant estimation of spontaneous mutation rate would be lower at rural than steel sites. This would result in an over-estimation of induced mutation (over spontaneous) at steel sites. Similarly, it has been established that one allele of the human minisatellite MS32 has a sequence mutation upstream of the repeat units that acts in cis to decrease the mutation rate (Monckton et al., 1994). The allele is present at high frequency in African populations compared to Caucasians and Japanese. These sorts of population differences emphasize need for careful scrutiny of control populations for minisatellite studies because intrinsic differences may exist between minisatellite instabilities in different populations.

Differences in the ages of the parents sampled might also explain differences in the minisatellite mutation rates observed because older parents might be more susceptible to germ cell mutations. Offspring of older parents could have a higher probability of showing a minisatellite mutation. Therefore, a biased sampling of older parents at some sites might result in increased mutation rates there. However, there is no evidence that older parents exhibit higher rates of minisatellite germline mutations. Therefore, age-related minisatellite germline mutagenesis should be investigated to determine whether older birds transmit more mutations.

1.5 The herring gull as a sentinel species

The herring gull, Larus argentatus, is predominantly a fish-eating bird and a long-lived resident of the Great Lakes Basin. Because herring gulls are ground nesting, colonial and monogamous, samples from complete families are easily obtained for DNA fingerprinting. Importantly, because they are non-migratory in the Great Lakes, their
overall exposures to chemical contamination can be directly attributed to exposures in their local environments, and not to exposures in other, possibly more contaminated areas to which they might migrate (e.g., Mexico). For these reasons, the herring gull has been used as a sentinel species by Canadian Wildlife Service and U.S. Fish and Wildlife Service scientists for more than twenty years.

1.6 Thesis Objectives

The primary objective of my thesis is to use DNA fingerprinting to test the hypothesis that germline minisatellite mutations are induced in herring gulls as a result of exposures to urban and industrial contaminants. More specifically, I focused on herring gull colonies situated near steel industries that operate coking ovens, major sources of environmental mutagens, compared to rural locations.

A second objective of my thesis was to investigate the two alternative hypotheses put forth to explain differences in minisatellite mutation rates including: (1) genetic differences in the subsets of minisatellite loci scored between the populations, and (2) age-related minisatellite mutagenesis, to investigate whether older parents may pass on more minisatellite mutations.

The final objective of my thesis was to estimate biologically relevant exposures to chemical mutagens by quantifying the levels of DNA adducts in herring gull erythrocytes using the $^{32}$P-postlabelling method. Compounds like polycyclic aromatic hydrocarbons, predominant in urban and industrial areas, are metabolized to reactive intermediates, and, if unrepaired, may induce DNA mutations. The primary goal of the DNA adduct study was to determine whether levels of DNA adducts are different between
the steel and rural sites, and whether these levels correlate with minisatellite mutation rates.

The overall goal of this work is to assess the suitability of multi-locus DNA fingerprinting as a biomarker for induced germline mutagenesis in organisms exposed to complex chemical mixtures *in situ*. Minisatellite DNA may provide a sensitive biomarker for measurement of induced germline mutations.
CHAPTER 2
Classification: Biological Sciences

Multi-locus DNA fingerprinting reveals high rate of heritable genetic mutation in herring gulls nesting in an industrialized urban site.

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Abbreviations: PACs, polycyclic aromatic compounds.

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ABSTRACT

Genotoxins, such as polycyclic aromatic compounds (PACs), are ubiquitous in urban and industrial environments. Our understanding of the role that these chemicals play in generating DNA sequence mutations is predominantly derived from laboratory studies with specific genotoxins or extracts of contaminants from environmental media. Most assays are not indicative of the germinal effects of exposure *in situ* to complex mixtures of common environmental mutagens. Using multi-locus DNA fingerprinting we found the mutation rate in herring gulls inhabiting a heavily industrialized urban harbour (Hamilton Harbour, Ontario) to be more than twice as high as three rural sites: Kent Island, Bay of Fundy, Chantry Island, Lake Huron and Presqu’ille Provincial Park in Lake Ontario. Overall we found a mutation rate of 0.017±0.004 per offspring band in Hamilton, 0.006±0.002 at Kent Island, 0.002±0.002 from Chantry Island and 0.004±0.002 from Presqu’ille Provincial Park. The mutation rates from the rural sites were significantly lower than the rates observed in Hamilton Harbour (Fisher’s exact, two-tailed, p=0.033). These minisatellite DNA mutations may be important biomarkers for heritable genetic changes resulting from *in situ* exposure to environmental genotoxins in a free living vertebrate species.
Levels of persistent genotoxic chemicals are elevated in the aquatic sediments and air of many areas. One possible consequence of exposure to these mutagenic compounds is an increase in germline mutations affecting the frequency of genetic diseases in a population. Certain chemicals have been shown to induce mutations in laboratory experiments. Ex situ assays are essential for establishing possible effects on species exposed to specific contaminants, but do not adequately evaluate the effects of complex mixtures encountered in nature. In situ techniques are typically indirect measures of mutagenicity (sister chromatid exchange, DNA adduct analyses) or measure significant biological endpoints that often result in spontaneous abortion (chromosome aberration) creating difficulty in observing significantly elevated levels in viable offspring (1). Most screenings for direct DNA sequence changes are somatic cell assays. For example, studies have shown increased mutation rates in smokers at the Hypoxanthine PhosphoRibosyl Transferase (hppt) locus (2) and in chemotherapy patients (3). However, even in acutely exposed individuals, mutations are extremely rare events and a large sample size is needed to obtain statistically significant results. In addition, these somatic assays do not reveal the possible implications of exposure to future generations.

It has been difficult to establish that certain agents induce heritable mutations. In the laboratory, mouse assays, such as the mouse dominant lethal test, are used to determine the genetic effects of mutagens (4). This evaluates mortality in the fetuses of mutagen-treated and untreated mice. A large number of animals are required to obtain significant results and the assay does not represent biologically relevant exposure to ambient concentrations of complex mixtures of chemicals.

Induction of germline mutations was examined recently in humans and animals acutely exposed to radiation. Baker et al. (5) estimated base-pair substitution rates for the mitochondrial cytochrome b gene in two free-living species of voles (Microtus arvalis and M. rossiaemeridionalis) inhabiting an area near reactor 4 at Chernobyl, Ukraine. They found rates of substitution (0.0002 nucleotides per site per generation) to be hundreds of
times greater than typical mitochondrial rates (normally $10^{-6}$ to $10^{-8}$; 6,7). Such high rates of mutation in a protein-coding gene have never before been documented in a mammalian species. However, substitution rates in mitochondrial DNA are much higher than nuclear genes, and may not reflect mutation in the nuclear genome (8). Kodaira et al. (9) examined children of atomic bomb survivors at six minisatellite loci using Southern blot analysis and observed no significant difference in mutation rates in children of exposed versus unexposed parents. Radiation has been shown to cause DNA alterations in F1 progeny of Japanese medaka fish (Oryzias latipes) at microsatellite loci (10), and at minisatellite loci in mice (Mus musculus; C57BL/6N, C3H/HeN and [101/HY X C3H/SnY] F1-hybrids; ref. 11,12) Dubrova et al. (12) noted a statistical increase in mutation frequency in offspring of mice exposed to 0.5 Gy $\gamma$-radiation. In a follow-up study Dubrova et al. (13) examined the DNA fingerprints of survivors of the Chernobyl accident and their more recently born children inhabiting a heavily polluted (radioactive and non-radioactive) area of the Mogilev district of Belarus. They found the frequency of mutations to be twice as high in the exposed families compared to a control group from the United Kingdom. The mutation rate in the Mogilev group correlated with exposure to caesium-137 indicating radiation induction of germ-line mutations. The authors suggest that environmental mutagens resulting from agricultural or industrial processes may also play a role in the induction of germ-line mutation in addition to post-Chernobyl radioactive contamination.

Here, we report the first use of multi-locus DNA fingerprinting to examine in situ mutations in families of herring gulls (Larus argentatus), a sentinel species, nesting in an industrial urban harbour contaminated with non-radioactive chemicals, and from relatively uncontaminated rural sites in the Great Lakes and in the Bay of Fundy. We show a significantly higher rate of mutation in gulls exposed to potentially hazardous chemicals. Herring gulls, long-lived fish eaters distributed throughout the Northern hemisphere, are non-migratory in the Great Lakes. Therefore, effects of contamination can be attributed
to their local environment and not to exposure during migration or on more polluted wintering grounds.

MATERIALS AND METHODS

Sampling sites. Hamilton Harbour (43°15' N, 79°51' W) is a natural embayment at the extreme western end of Lake Ontario. The Harbour is the site of the two largest steel mills in Canada. The water, sediment and the air in the Hamilton area are known to be contaminated with PACs and other chemicals showing genotoxic activity (14, 15) including heavy metals (16). Chantry Island on Lake Huron (44°29' N, 81°23' W) and Presqu'ile Provincial Park on Lake Ontario (44°00' N, 77°43' W) are two clean rural colonies within the Great Lakes. These colonies are removed from the mainland and are not situated near point sources of PAHs from roads or industries. Kent Island (44°34' N, 66°45' W), the site of our “pristine” control colony in the Bay of Fundy, is also removed from the mainland and any point sources of PAHs.

Sample collection. Adult herring gulls were captured on their nests late in the incubation period, in 1992 and 1993. Small-volume blood samples were collected from the brachial vein of adults and from the jugular vein of chicks within two days of hatching. Blood was stored in 1X lysis buffer (4.0M Urea; 0.2M NaCl; 0.1M Tris-HCl, pH 8.0; 0.5% n-laurylsarcosine; 0.1M CDTA [1,2-cyclohexanediamine]) at 4°C. Our handling of gulls was in accordance with University animal care guidelines. We analyzed DNA from 35 nestlings and their parents from Hamilton Harbour, 47 from Kent Island, 32 from Chantry Island and 27 from Presqu’ile Provincial Park.

Molecular analyses. Approximately 25-50 µl of blood was digested twice with proteinase K (83.3 units at 37°C for 12 hours) and purified with two phenol/chloroform
(70:30) and one chloroform extraction (17). Concentrations of DNA were determined with a TK100 mini-fluorometer and with agarose gel electrophoresis.

Following quantification, 15 μg of herring gull DNA was digested with HaeIII and ethanol precipitated. Three nanograms of digested λ DNA size marker (18) combined with 4.5 μg of sample DNA were size fractionated by electrophoresis for 36-48 hours at 1.25 to 1.5 volts/cm in 28 cm long, 0.8% agarose gels. BamH1 digested adenovirus size standards were run as side lane markers. Following electrophoresis, the gels were acid nicked for 15 minutes (0.25M HCl), denatured (1.5M NaCl; 0.5M NaOH) for 1 hour and neutralized (1.5M NaCl; 0.5M Tris base; 1M EDTA [disodium ethylene diamine tetraacetate•2H2O]; pH 7.2) for 1 hour. DNA was transferred to a polyvinylidene membrane (Immobilon N, Millipore) by Southern blotting, then air dried and baked to fix the DNA to the membrane (1-2 hours at 80°C).

Blots were prehybridized at 65°C with a sodium orthophosphate prehybridization solution (19). The blots were sequentially probed with 4 probes: Jeffreys 33.15, J33.6 (20), pSP2.5RI (PER) mouse probe homologous to the Drosophila periodic gene (21) and finally λ. Unincorporated nucleotides were separated from labeled probe with a sepharose spin column. After overnight hybridization, blots were washed (2X SSC, 0.1% SDS) at 65°C and placed on X-ray film at -70°C for both short and long exposures. After stripping, the blots were again exposed to film for a minimum of 48 hours to ensure that probe DNA was removed before subsequent probing.

Analysis. For each colony we quantified the number of novel bands (not found in either parent's sample) between 23 and 3.5 Kb in each nestling's DNA profile. The DNA fingerprints were scored without knowledge of colony location. Although herring gulls are socially monogamous, we did not make assumptions about parentage. The probability of only 1 novel band due to extra-pair fertilization is extremely low given the hypervariability of the loci and
the number of loci examined per individual. These bands were considered the result of mutations. Average band sharing was calculated to be 0.18 based on pair-wise comparisons of 35 randomly selected adults from two colonies (Band sharing = 2 x no. of bands shared / total no. of bands scored; 22). Two chicks, each with 12 non-maternal bands, were found to have two novel bands each. The probability that a randomly selected adult (representing an extra-pair copulation) would share 10 or more of these bands was calculated to be $1.6 \times 10^{-6}$ ($\sum_{i=1}^{11} i * 0.18^{10} * 0.82^2 + 12 * 0.18^{11} * 0.82 + 0.18^{12}$) suggesting that the novel bands were mutations rather than fragments inherited from an extra-pair fertilization. Band-sharing between parent and offspring for nestling exhibiting one or two novel fragments was high (0.44-0.80; mean =0.58±0.08) Five nestlings examined exhibited 6 or more novel bands and were not included in the analysis. One nestling from Kent Island had a mutation that was detected by both J33.15 and J33.6, and one nestling from Chantry Island had a mutation that was detected by all three of the probes used. These mutations were counted as one mutational event in the determination of overall mutation rate, but were included with each probe in the calculation of mutation rate per probe. A sub-sample (70%) of nestlings with mutations from Hamilton Harbour were re-run to confirm that the mutant fragments were reproducible. In each case mutations were present within the same size range.

A two-tailed Fisher’s exact test was used to compare the mutation rate for Hamilton Harbour compared to each of the cleaner sites.
RESULTS

A well-spaced banding pattern allowed the easy detection of novel bands (bands arising from length change mutations) from DNA fingerprints of herring gull families (Fig. 2.1). We identified 16 novel bands in samples from 14 individuals of the 35 nestlings examined from Hamilton Harbour, 3 novel bands from 3 nestlings of the 29 individuals sampled at Presqu'ile Provincial Park, 2 mutations in 32 nestlings from Chantry Island, and 7 novel fragments from 7 individuals, of the 47 examined from Kent Island (Table 2.1). The mutation rate (mutations per fragment scored per generation) in Hamilton was significantly higher than the mutation rates calculated for Kent Island, Chantry Island and Presqu'ile Provincial Park (Fisher's exact test, two-tailed; $p=0.018$, $p=0.003$ and $p=0.032$ respectively). The induced mutation rate, possibly linked to chemical contamination, was found to be 0.011 between Hamilton Harbour and Kent Island (the highest rate found among the control colonies). Differences among clean colonies were not statistically significant (Fisher's exact; $p=0.328$ to 0.994). The mutation rates per fragment scored at the clean sites are similar to the rates found in other species, including humans (Table 2.2).

DISCUSSION

Herring gull nestlings from Hamilton Harbour, an extensively industrialized urban site, exhibited a significantly higher rate of mutation than three non-industrial rural locations. The increased mutation rate may be explained by elevated concentrations of genotoxins in the aquatic sediments and air particulate at the Hamilton site. The Harbour is known to be contaminated with chemicals showing high levels of genotoxic activity. Polycyclic aromatic compounds (PACs), predominant genotoxins in urban and industrial areas, are abundant in the air in Hamilton and in the sediment of Hamilton Harbour (14,15), and concentrations of heavy metals are elevated in the Harbour sediments (16). Hamilton Harbour herring gulls are exposed to contaminants from two possible sources:
(1) airborne contaminants from coking process emissions from the steel industries and/or from vehicle emissions from a nearby heavily used highway complex, and (2) chemical contaminants in their aquatic diet. Conversely, the rural Great Lakes sites, Presqu'ile Provincial Park and Chantry Island, are removed from point sources of contaminants such as heavy industries and highway complexes. Kent Island, our "pristine" colony in the Bay of Fundy, is distant from the mainland and any point sources of contaminants. Concentrations of polychlorinated biphenyls and other organochlorines in the eggs and tissue of gulls from Kent Island have been approximately one fifth the concentration here than all other herring gull colonies analyzed in the Great Lakes for the past 15 years (26). For this reason, Kent Island has been used routinely by the Canadian Wildlife Service as a control site for toxicological studies. We suggest that the current ambient levels of chemical contaminants present in the environment of the Hamilton Harbour herring gulls may be inducing significantly elevated rates of minisatellite DNA mutations.

Currently we are investigating two competing hypotheses to explain differences in the mutation frequencies observed. First, mutation rates in Hamilton Harbour may be elevated if the average age of adults sampled is greater than at other colonies, since older individuals may be more susceptible to mutation. Second, differences may be attributed to a highly mutable subset of alleles limited to the Hamilton Harbour population. There is a possibility that age or allelic differences may exist between Hamilton Harbour and Kent Island given the great distance between them. It is unlikely that such differences will be true for colonies within the Great Lakes since much more mixing should occur in this small area, and climatic conditions are very similar. We are using band sharing analyses of DNA fingerprints from randomly selected adult herring gulls from each of the colonies to determine whether birds from different colonies within the Great Lakes are from the same genetic stock. In addition, we are investigating age-related mutation rates in a group of known-aged herring gulls from Presqu'ile Provincial Park. We are currently sampling
gulls from other industrialized urban sites to generalize the effects of high concentrations of ambient contaminants on minisatellite DNA mutations.

It has been shown in laboratory experiments that chemical contamination affects the rate at which minisatellite mutations arise in vitro. Kitazawa et al. (27) have shown that 2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-b] pyridine (PhIP) can induce fragment-length change mutations in two mouse tumor cell lines using multi-locus DNA fingerprinting. They found that length change mutations arose at frequencies between 21-53% and 22-35% (control = 0%), depending on the concentration of PhIP, for the two lines respectively and concluded that PhIP induces recombinational mutations. Ogheri et al. (28) examined the induction of minisatellite DNA mutation in V79 cells treated with methyl-nitro-nitroso guanidine (MNNG). They compared MNNG-induced mutation frequencies at the hprt locus to minisatellite DNA mutation frequencies examined with the J33.15 probe. They concluded that DNA mutations occurred at minisatellites much more frequently than in transcribed sequences (4%-31% minisatellite mutant frequency for different concentrations of MNNG-treated cells, versus 1.6%-7.5% hprt mutant frequency). A statistically significant difference in the frequency of minisatellite mutations between MNNG-treated cells and their controls was seen. Ledwith et al. (29) found a 2- to 5-fold higher frequency of minisatellite DNA rearrangements in chemical carcinogen-induced mouse liver tumors compared to spontaneous tumors. They suggest that DNA fingerprinting may be used to differentiate between chemically-induced versus spontaneous tumors. Dubrova et al. (13) showed a doubling in germinal minisatellite mutations induced by radiation in Chernobyl families compared to families from the United Kingdom (Table 2). The control families in this study had rates of mutation similar to those found in the Hamilton Harbour herring gull families and higher than previously reported rates of mutation in humans with the same probes (25). The authors suggest that non-radioactive contaminants present in the environment of the Chernobyl survivors may also play a role in the induction of minisatellite mutations. Ubiquitous environmental
mutagens may elevate background mutation frequencies in humans inhabiting urban areas like the United Kingdom.

Minisatellite DNA examined with DNA fingerprinting has several advantages for the detection of genotoxin-induced mutagenesis. These tandemly-repeated arrays of nucleotides show high rates of mutation compared to unique sequence DNA (30,31), probably due to unequal sister chromatid exchange, gene conversions or replication slippage (32,33). Such extremely variable minisatellite DNA loci may be hypersensitive to induced instability, thereby reducing the sample sizes needed for mutation analyses (34). Furthermore, the mutations seen with DNA fingerprinting are predominantly heritable (35). Several studies have shown somatic stability of minisatellite DNA (18) and inheritance of mutant fragments in subsequent generations in humans (30). Therefore mutations detected by minisatellite DNA analyses likely occur in the germ-line or possibly very early in embryogenesis, before differentiation of somatic and germ-line tissues. Additionally, by using multi-locus DNA fingerprinting we can survey several loci simultaneously, thereby increasing the statistical power to distinguish mutation rates among different sites. Finally, analyses of minisatellite DNA should be less affected by selection as minisatellites are generally in non-coding regions of the genome.

Understanding the mutagenic impact of ambient concentrations of pollutants on germ cells is of immediate concern as heritable changes, as well as somatic changes, may result in increased genetic disease. The factors responsible for elevated levels in Hamilton Harbour may vary with environmental conditions (e.g. air pollution levels vary with weather) and causal relationships remain to be determined. The higher mutation frequency in the Hamilton Harbour herring gulls is consistent with the degree of industrial contamination present at this location. Because the mutations were measured in a higher vertebrate, possessing complex enzymatic systems for the metabolism of xenobiotics, all members of this ecosystem, including humans, are potentially at risk. These heritable
sequence changes appear to be relevant biomarkers that can be monitored in free-living species in situ.

ACKNOWLEDGEMENTS

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REFERENCES


Figure 2.1 DNA fingerprints of samples from three herring gull families from Hamilton Harbour digested with HaeIII and probed with J33.15. The three novel bands are indicated with arrows. F and M designate the Female and Male parents. N1 to N3 designate the nestlings (one nestling in family 2 was sampled twice).
Table 2.1. DNA fingerprinting mutation rates in herring gull nestlings.

<table>
<thead>
<tr>
<th>Location</th>
<th>No. of mutations</th>
<th>No. of nestlings scored</th>
<th>No. fragments scored</th>
<th>Mean No. Bands Scored ± S.E.</th>
<th>Mutation rate* per fragment scored</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hamilton Harbour</td>
<td>16 (14)</td>
<td>35</td>
<td>969</td>
<td>27.7±0.9</td>
<td>0.017±0.004</td>
</tr>
<tr>
<td>Kent Island</td>
<td>7 (7)</td>
<td>47</td>
<td>1252</td>
<td>26.6±0.8</td>
<td>0.006±0.002</td>
</tr>
<tr>
<td>Chantry Island</td>
<td>2 (2)</td>
<td>32</td>
<td>844</td>
<td>26.4±0.9</td>
<td>0.002±0.002</td>
</tr>
<tr>
<td>Presqu'ile Park</td>
<td>3 (3)</td>
<td>29</td>
<td>688</td>
<td>23.7±1.0</td>
<td>0.004±0.002</td>
</tr>
</tbody>
</table>

*Rate = # novel bands / total # bands scored

‡ SE = \( \sqrt{\frac{p(1-p)n}{n}} \)
Table 2.2 A comparison of minisatellite mutation rates per fragment scored for various species.

<table>
<thead>
<tr>
<th>Species</th>
<th>Mutation rate (probe)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Larus argentatus</em> (Hamilton Harbour)</td>
<td>0.019±0.007* (J33.15)</td>
<td>THIS STUDY</td>
</tr>
<tr>
<td></td>
<td>0.019±0.007 (J33.6)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.009±0.006 (PER)</td>
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<tr>
<td><em>Larus argentatus</em> (Kent Island)</td>
<td>0.006±0.004 (J33.15)</td>
<td>THIS STUDY</td>
</tr>
<tr>
<td></td>
<td>0.007±0.004 (J33.6)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.006±0.004 (PER)</td>
<td></td>
</tr>
<tr>
<td><em>Larus argentatus</em> (Chantry Island)</td>
<td>0.006±0.004 (J33.15)</td>
<td>THIS STUDY</td>
</tr>
<tr>
<td></td>
<td>0.003±0.003 (J33.6)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.004±0.004 (PER)</td>
<td></td>
</tr>
<tr>
<td><em>Larus argentatus</em> (Presqu’ile Park)</td>
<td>0.012±0.007 (J33.15)</td>
<td>THIS STUDY</td>
</tr>
<tr>
<td></td>
<td>0.000 (J33.6)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.000 (PER)</td>
<td></td>
</tr>
<tr>
<td>House Sparrow <em>Passer domesticus</em></td>
<td>0.004 (J33.15, J33.6)</td>
<td>(23)</td>
</tr>
<tr>
<td>Great Tit <em>Parus major</em></td>
<td>0.008 (J33.15, M13)</td>
<td>(24)</td>
</tr>
<tr>
<td>Blue Tit <em>Parus caeruleus</em></td>
<td>0.003 (J33.15, M13)</td>
<td>(24)</td>
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<td>Humans <em>Homo sapiens</em></td>
<td>0.011 (J33.15)</td>
<td>(25)</td>
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<td>0.05 (J33.6)</td>
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<tr>
<td></td>
<td>0.0303 (J33.15, MS32, CEB1)</td>
<td>(10)</td>
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SE = |p(1-p)/n|^{0.5}
CHAPTER 3
Monitoring for induced heritable mutations in natural populations: application of minisatellite DNA screening
[Mini-review]

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Abstract

The need to understand the role that anthropogenic chemicals play in generating germ-line mutations is critical, both from an ecological and a human health perspective. Exposure to complex mixtures of urban and industrial chemicals is widespread and we have little understanding of the long-term implications to populations and gene pools. It has recently been suggested that minisatellite DNA mutations may be sensitive biomarkers for induced heritable mutations in populations exposed to radioactive and non-radioactive contamination in their environments. Minisatellite loci are attractive targets for mutational analyses because they undergo a rate of mutation much greater than unique sequence DNA and with DNA fingerprinting many loci can be scanned simultaneously. As a result, the technique is statistically powerful requiring relatively small sample sizes (compared to other in situ mutation assays) and is reasonably cost and time efficient. This paper will review the application of minisatellite mutation screening to the field of genetic toxicology.

Keywords: biomarker; heritable mutation; minisatellite DNA; genotoxicology

1. Introduction

Anthropogenic activities have resulted in widespread distribution of contaminants, some of which are genotoxic. Our knowledge of the distribution of these chemicals, many of which persist for long periods of time in our environment, and the potential adverse effects they pose on natural gene pools is very limited. Organisms in nature are exposed to hundreds, even thousands of mutagenic chemicals simultaneously and temporal exposure may vary tremendously. Evaluation of the heritable genetic effects of chemicals
present in our ecosystem is problematic because mutations are extremely rare, typically rendering statistical comparisons between exposed and unexposed populations insignificant. Furthermore, the nature of exposure to complex mixtures of chemicals in situ makes causal links between exposure and mutation difficult to demonstrate in natural populations.

Recently our understanding of the mutagenic activity of components of complex chemical mixtures found in our urban environment has increased substantially through application of bioassay-directed fractionation. This technique fractionates components of chemical mixtures sampled from environmental media like air, water or aquatic sediment, and determines the mutagenic potential of the fractions with standard laboratory mutation assays, like the Ames test [1, 2]. Bioassay-directed fractionation is a useful technique to identify chemicals present in our environment, generated from numerous sources, that have the ability to induce mutation [e.g. 3, 4]. Classes of chemicals contributing the majority of mutagenicity to environmental samples can be identified, thereby determining sources of potential risk necessary to establish remedial/legislative objectives. Isolated individuals or organisms that occupy a niche with the potential for greater exposure to a source of mutagens can be targeted for further study. However, ex situ assays that identify the presence of mutagens in the environment can not reveal the direct mutagenic impact of complex mixtures on populations of organisms exposed in the field to ambient concentrations of “chemical soups.” Additionally, these laboratory assays can never evaluate risk posed across taxa.

DNA damage induced by environmental contaminants occurs at many levels of organization in the genome including: the chromosome, the gene or the nucleotide. Assays are available to quantitate and characterize damage at each of these levels. In spite of this analytical capability, it has been difficult to show a relation between anthropogenic chemicals found in nature and induction of germ-line mutations. Typical assays used in the field to estimate genetic risk are often somatic assays and/or indirect measures of
mutagenicity. For example, DNA adducts and DNA strand breaks can be measured in organisms to demonstrate exposure to mutagens in the environment capable of binding to DNA [e.g. 5, 6, 7] or inducing strand breakage [e.g. 8, 9] and therefore potentially able to induce mutations. These measures do not reveal direct DNA sequence changes that may be transmitted to offspring. Cytogenetic assays can be used to measure large chromosomal changes, like chromosome aberrations, micronuclei induction or aneuploidy [10]. Certain types of chromosomal damage have been directly linked to adverse health effects and are therefore critical to evaluate in terms of environmental exposure. However, these large chromosome alterations do not examine mutations that may be occurring at the DNA sequence level.

With the advent of numerous molecular techniques much progress has been made in the analysis of induced DNA sequence mutations. Mutations arising at the nucleotide level include: base-pair substitutions (transitions, transversions), frameshifts resulting from small deletions or insertions, or large scale genomic rearrangements like deletions, insertions, duplications, translocations and inversions. These types of genomic changes occurring in situ have mostly been studied in human somatic cells at specific loci, and particular attention has been paid to the hypoxanthine phosphorybosyl transferase (hprt) locus on the X chromosome. Elevated mutation rates at the hprt locus have been found in human somatic cells of individuals exposed to: acute radiation [11, 12], anticancer treatments [13, 14], or in specific occupational settings [15, 16]. The only study to show elevated germ-line sequence mutations at a coding locus examined mitochondrial DNA in voles exposed to radioactive contamination. Baker et al. [17] estimated base-pair substitution rates for the cytochrome b gene in two free-living species of voles (Microtus arvalis and M. rossiaemeridionalis) inhabiting an area near reactor 4 at Chernobyl, Ukraine. They found rates of substitution (2 X 10^-4 nucleotides per site per generation) to be hundreds of times greater than typical mitochondrial rates (normally 10^-6 to 10^-8; ref. 18, 19) and significantly higher than voles from a control populations 32 km southeast
of the reactor. Such high rates of mutation in a protein-coding gene have never before been documented in a mammalian species and warrant further investigation. However, substitution rates in mitochondrial DNA are much higher than nuclear genes, possibly due to differences in DNA repair or protein/DNA complexes, and may not reflect mutation in the nuclear genome [20, 21].

Screening of mutations induced by chemicals at specific loci has revealed that the mutation rate throughout the genome is far from homogenous. Local patterns in the types and frequency of mutation (mutational spectra) can result from differences in the composition and structure of the protein-DNA complex. For example, short stretches of replicating DNA, or units of linker DNA between nucleosomes, might be more susceptible to binding of adduct-forming chemicals [reviewed in 22]. Other factors contributing to the heterogeneity of mutation rates along the genome include: DNA regions that are transcribed versus non-transcribed, coding versus non-coding DNA, functional (involved directly or indirectly in transcription or translation) versus 'junk' DNA, or the intrinsic nature of the DNA sequence itself [23]. Local 'hot spots' of mutation have been detected revealing unique mutational spectra linked to specific chemical exposures (both single chemical, ref. 24, 25, 26; and complex mixtures, ref. 3) at particular loci. Screening areas known to have high rates of mutation will increase the probability of detecting altered genotypes in offspring of parents exposed in the field.

Analysis of the vertebrate genome has revealed sequence elements known as minisatellite DNA that are subject to very high rates of germinal mutation [27]. Minisatellite DNA sequences are tandemly repeated arrays of nucleotides, with units varying between 10 to 100 base pairs in length, that are spread throughout the genome of eukaryotic organisms. In humans, minisatellite DNA sequence variability has been extensively studied. These regions show extreme polymorphism both in repeat copy number [27, 28] and in the pattern of variant repeat unit organization along the tandem array [29], with average mutation rates as high as 0.06 per gamete in humans having been
documented [30]. Minisatellite mutation rates are much higher than rates in unique sequence DNA regions considered to be hypervariable. For example, the mitochondrial DNA control region has a mutation rate of 1.2 to $27.0 \times 10^{-6}$ per site per generation in humans and is one of the most variable unique sequence regions known [31]. The hypervariability at minisatellite loci is maintained through a high germinal mutation rate producing new length alleles resulting from changes in the number of repeated units. Mutations are identified as novel fragments present in the DNA fingerprints of offspring that can not be ascribed to either parent (Figure 3.1). Because minisatellites are predominantly in non-coding regions of the genome, mutations are not manifested as malformations or abortion. These biases may present statistical problems with screening for mutation at coding loci, or for gross chromosomal aberrations which frequently result in spontaneous abortion [32]. The recent use of DNA fingerprinting in full families of humans [33, 34] and birds [35] has led to the first demonstrations of statistically significant induced heritable mutagenesis resulting from exposure to contaminants in situ. Here I review the application of minisatellite DNA as a biomarker for induced heritable mutagenesis.

2. Minisatellite DNA

Minisatellite DNA sequences were first shown to exhibit high rates of germinal mutations in studies of human pedigrees using multi-locus DNA fingerprinting [28]. Analyses of mutation rates in the two most hypervariable minisatellite loci found in humans, λMS1 [27] and CEB1 [30], reveal average mutation rates of 5% and 6% per gamete respectively. Parental origin of minisatellite DNA mutations (maternal versus paternal) is locus-specific despite differences in the number of mitotic cell divisions in the germline leading to sperm and oocytes. For example, analysis of the human minisatellites MS32 and MS205 show mutation ratios of 1:1 and 11:1 for male to female germlines respectively [36]. At the hypervariable locus CEB1 in humans, 13% of sperm show
mutations versus 0.3% of oocytes [30].

Powerful new approaches have been developed for the analysis of de novo mutations arising in pedigrees (minisatellite variant repeat mapping by PCR; MVR-PCR; ref. 37) and for the analysis of sperm samples (small pool PCR; SP-PCR; ref. 29). With these techniques, the re-arrangement of variant repeat units along the tandem array in de novo mutations are examined in order to elucidate possible mechanisms of mutation. Minisatellite mutations may arise through a number of different processes including unequal recombination at meiosis, unequal sister chromatid exchange, DNA polymerase slippage at replication forks and gene conversion. Analyses of some single locus human minisatellites show mutations containing complex rearrangements without exchange of flanking sequences, suggesting that gene conversion events, rather than unequal recombination at meiosis or DNA polymerase slippage, might be the predominant mechanism acting at these loci [29, 38]. Gene conversions are intra- or inter-strand recombinational events that result in a preferential gain in minisatellite repeat units. It is hypothesized that double strand nicks in the DNA template is followed by strand invasion of the complementary allele, either from the sister chromatid or from the homologous chromosome, to produce new length minisatellite sequences. Although double strand breaks are required for gene conversion events, studies have shown that the induction of double strand breaks by radiation at minisatellite loci is not the mechanism generating increases in minisatellite mutations. Analyses of germinal mutation rates in mice exposed to 60Co-γ-irradiation showed higher levels of minisatellite mutation than would be expected from the predicted frequency of strand breaks per locus, suggesting that another (unknown) mechanism is operating in induced minisatellite instability [39]. Scoring of minisatellite mutation events following exposure to radiation reveals the indirect impact of radiation elsewhere in the genome and may include gap repair as an important step in the process [33]. It has also been suggested that minisatellite binding proteins (Msbp; ref. 40, 41) may be important in the induction of mutations at minisatellite loci [39, 42].
study on the induction of minisatellite mutations after exposure to okadaic acid (OA), Nakagama et al. [42] noted a significant increase in mutation rate in exposed NIH 3T3 cells. OA is a strong tumor promoter and an inhibitor of serine/threonine protein phosphatases [43, 44, 45]. Because there has been no evidence that OA interacts directly with the DNA, Nakagama et al. suggest that perturbation of the phosphorylation status of proteins could be a contributor to induced minisatellite instability. They suggest that alterations in the phosphorylation of Msbp's may lead to changes in recombination machinery and consequently result in minisatellite mutations.

Mutant alleles of minisatellite DNA can arise in somatic cells as a result of mutation and clonal expansion. This can occur in cell lines and in tumors [46, 47]. Thus, DNA mutations that arise in malignancies and in cell lines exposed to genotoxic chemicals can be detected as changes in minisatellite DNA composition. In various tumor types, minisatellite mutations arise at a frequency significantly greater than in normal tissue [46, 47, 48, 49, 50]. Examination of cell lines has shown that non-radioactive chemicals with mutagenic potential can induce minisatellite length change mutations. Kitazawa et al. [51] showed that two mouse tumor cell lines exposed to 2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-b] pyridine (PhiP) produced length change mutations at a significantly higher frequency than their control cells. They found minisatellite mutations at frequencies between 21-53% and 22-35% for cells exposed to different doses of PhiP versus a 0% mutation rate in control cells. Oggeri et al. [52] showed that Chinese hamster V79 cells treated in vitro with methyl-nitro-nitroso guanidine (MNNG) had a statistically significant increase in the frequency of minisatellite mutations compared to untreated controls. When they compared minisatellite mutation to mutation at the hprt locus, they found that similar MNNG concentrations induced a 4-31% minisatellite mutant frequency but only a 1.6-7.5% mutant frequency within the transcribed portion of the hprt gene. This could indicate a hyper-sensitivity of minisatellite sequences to mutation by genotoxic chemicals compared to unique protein coding sequence DNAs. The fact that genotoxic chemicals
induce higher frequencies of minisatellite mutation has led to the application of DNA fingerprinting and single locus minisatellite analysis for the determination of germinal mutation induction in populations of animals exposed to mutagenic chemicals \textit{in situ}.

Multi-locus DNA fingerprinting has demonstrated significantly elevated mutation rates in the offspring of humans inhabiting the radioactive polluted areas of Belarus near Chernobyl, Ukraine [33, 34] and in herring gulls from an industrialized urban area [35] (Table 3.1). Dubrova \textit{et al.} [33] applied multi-locus DNA fingerprinting to the analysis of human families from Mogilev still living in the highly polluted rural areas of Belarus. These families received acute doses of iodine-131, and continue to be exposed to caesium-137. Minisatellite mutation rates from children of parents who were continuously resident in the Mogilev district from the time of the Chernobyl accident were compared with families in a control group from the United Kingdom. The rate of mutation among the exposed families was twice the rate for the control group. Dubrova \textit{et al.} [33] suggest that the significantly increased rate of germ-line mutation correlates to caesium-137 exposure and possibly other environmental mutagens in this polluted area. Questions have been raised regarding the appropriateness of the British control group used in this study because of population and environmental differences [53]. Intrinsic differences may exist between the minisatellite instabilities in these two populations. Variables such as genetic differences between populations need to be thoroughly investigated in relation to minisatellite mutation rate. It has been established that one allele of the minisatellite MS32 has a sequence mutation upstream of the repeat units that acts in \textit{cis} to decrease the mutation rate [54]. The allele is present at high frequency in African populations compared to Caucasians and Japanese. These sorts of population differences emphasize the careful scrutiny of control populations for minisatellite studies and the requirements for screening at multiple independent loci. To address this concern Dubrova \textit{et al.} [33] examined overall band sharing and allele distribution in the two populations and found no significant differences. Additionally, significantly higher mutation rates in the Mogilev
group were seen with three groups of probes scoring independent minisatellite loci, suggesting that the increased mutation rate could not be attributed to a single locus that has accumulated unusually unstable alleles in the Mogilev population. Finally, by examining relative exposure to surface contamination of caesium-137 in the Belarus group, they were able to show a significant dosage effect of radiation exposure on observed mutation rate.

In a follow up study, Dubrova et al. [34] expanded their research to include 48 more families from the contaminated regions and used 5 additional minisatellite probes (4 single locus, one multi-locus). The results from their original study remained unchanged; the mutation rate in the Belarus families was significantly elevated compared to families from the United Kingdom. A comparison of maternal and paternal ages revealed no significant differences in sampling between the populations, but information regarding the smoking status of the subjects was not available in either of the Belarus studies. Smoking is obviously a critical variable in mutation studies in humans and future research should match for both age and smoking when that information is available. Determination of allele size and overall minisatellite variability between the control group and the Belarus groups suggest that negligible differences exist in the genetic structure of minisatellite alleles in the two populations. Furthermore, the authors refined their estimates of parental exposure to radioactive contaminants by calculating individual radiation doses for chronic internal and external exposure to $^{137}$Cs through individual diets from the different localities of the Mogilev district, and through family history. Families within the exposed group were then divided into more exposed (>20mSv) and less exposed (<20mSv) subgroups and the mutation rates were determined (0.0238 and 0.0177 per band respectively for all probes combined). The mutation rate in the more exposed group was 1.35 times higher than the less exposed group ($p=0.0154$) and both groups were statistically significantly higher than the control group. These data provide compelling evidence for a link between radiation exposure and induced minisatellite mutation within
the exposed population. However, although a correlation between $^{137}$Cs and other radionuclides exists, the overall gonadal exposure for these individuals is unknown. Therefore, these data are not adequate for a detailed dose-response evaluation of radiation-induced minisatellite mutation. More research is needed to determine the dose-response relationship between radionuclide exposure and minisatellite mutagenesis.

Analysis of single locus minisatellite markers in human survivors of Hiroshima and Nagasaki and their offspring has not been able to detect increased germ-line mutation in this exposed group [55]. The extent and diversity of the exposure resulting from the atomic bombings is very different from Chernobyl and predictions drawn from one group are not likely to be very meaningful to the other [56]. The atomic bombings deal with a single acute administration of radiation compared to the Chernobyl region where individuals received high doses of acute $^{131}$I but are chronically exposed to $^{137}$Cs and other more stable isotopes. Kodaira et al. [55] examined 6 single locus minisatellite probes in 64 offspring born more than ten years after the bombings. Therefore, the gametes were assumed to be exposed at the spermatogonial or oocyte stage. Differences may exist in minisatellite sensitivity to radiation at different stages of spermatogenesis and oogenesis based on mouse studies [38, 57, 58] which might account for some differences in the results seen between the Chernobyl and A-bomb exposed families. Finally, Kodaira et al. [55] estimated that for a locus with a spontaneous mutation rate of 0.02 per gamete (the mean spontaneous mutation rate of the six loci scored in their study), an additional 13 loci would need to be scored in order to detect a statistically significant difference between the exposed and unexposed parents. They stated that this number was practical and that this population warrants further investigation with multi-locus DNA fingerprinting probes.

In our own investigations we examined colonies of herring gulls, a resident water bird of the Great Lakes [35]. These birds breed in areas that differ in amounts of non-radioactive industrial pollution. We determined that herring gull nestlings from Hamilton Harbour, a heavily industrialized urban harbor with a high concentration of steel industry,
had significantly higher rates of heritable minisatellite mutations than herring gull nestlings from areas of the Great Lakes with low inputs from anthropogenic sources and from a rural colony in the Bay of Fundy (Table 3.1). We suggested that the minisatellite mutation rate may be a sensitive biomarker for the induction of heritable mutation in this free-living, higher vertebrate species exposed to ambient levels of complex mixtures of chemicals. This is the first time that a significant increase in induced heritable mutation in a wild animal species exposed to "normal" urban levels of genotoxins has been shown. The herring gulls are exposed to genotoxic chemicals through contaminants present in their aquatic diet (they are predominantly fish-eating birds) and through inhalation of industrial and urban air pollutants. The unique respiratory system of birds results in a large mass-specific gas uptake at rest, and especially during exercise, suggesting that birds may represent a valuable sentinel for the study of in situ exposure to air pollution [reviewed in 59]. Possible age and allelic differences in the populations are currently being investigated in these populations. Age concerns are difficult to address in herring gulls because gulls can not be aged beyond their first five years. We have no reason to suspect that we have sampled older individuals at the Hamilton site. We are currently investigating mutation rates in a known sample of older parents from a rural location to determine whether minisatellite mutation rate increases in herring gulls with parental age. It is possible that allelic differences may exist between herring gull populations within the Great Lakes compared to the East Coast because little genetic exchange occurs between these locations. Overall band-sharing analyses of randomly selected adult herring gulls from Hamilton Harbour and from the Bay of Fundy revealed higher variability within the Hamilton colony (Table 3.2). Band-sharing coefficients compared between the colonies (compared to band-sharing coefficients within a colony) were not significantly different for two of the probes used (J33.6, PER) but were significantly lower between the colonies for the probe J33.15 suggesting that genetic differences might exist between these two locations (in preparation). It is unlikely that these differences will exist for colonies
sampled within the Great Lakes since much more genetic exchange should occur between these sites. We are currently investigating band-sharing and mutation rate for other colonies we have sampled in the Great Lakes. Preliminary data collected from one other site known to be contaminated with industrial chemicals show that herring gulls nesting in East Chicago, Indiana, USA, the site of the highest concentration of steel industry on the Great Lakes, exhibit mutation rates similar to Hamilton Harbour nestlings and significantly higher than our Great Lakes rural sites (in preparation). These findings support the link between induced heritable minisatellite mutation and exposure to complex mixtures of genotoxins from industrial and urban areas.

Minisatellite DNA has several advantages for the detection of genotoxin-induced mutation: (1) minisatellite DNA has a much higher rate of mutation than unique sequence DNA; (2) mutations are predominantly germ-line in origin; (3) by using DNA fingerprinting several loci are screened simultaneously increasing the probability of detecting mutations and therefore increasing statistical power and reducing the time and cost needed to screen for germ-line mutations; (4) minisatellites are predominantly in non-coding regions of the genome; therefore analyses of such regions are not affected by selection as mutations in minisatellites are not phenotypically expressed.

Although minisatellites are predominantly in non-coding regions, a number of minisatellites are associated with important genes and several play roles in the regulation of transcription with these associated genes including: the *HRAS1* proto-oncogene [61], the *IDDM2* gene associated with type 1 diabetes [62, 63], the human serotonin transporter gene [64] and myoclonus epilepsy type 1 [65]. Increased minisatellite DNA mutations could be important indicators that are reflective of genotoxic and disease-related risk.
3. Future Directions

The recent application of minisatellite markers to genetic toxicology suggest that these regions may be powerful biomarkers for mutation induced by contaminants found in the environment. However, before minisatellite markers can be implemented in biomonitoring, more research is needed. Dose-response relationships need to be established for radionuclides and non-radioactive contamination resulting from anthropogenic sources in order to clarify mutation induction in relation to exposure. No laboratory studies to date have examined dose-response relationships in germ-line minisatellite mutation in vivo for chemical exposures. The impact of acute versus chronic exposure to mutagens on minisatellite mutation rates needs to be critically assessed. As in any genetic study, confounding factors such as age and smoking need to be critically examined for biomonitoring applications. Variables such as genetic differences between populations need to be thoroughly investigated in relation to minisatellite mutation rate. Population differences should be carefully scrutinized for minisatellite studies and screening at multiple independent loci should be undertaken. Further investigation into the correlation between minisatellite mutations and mutations in other regions of the genome, including loci which may be pertinent to health and reproduction, should be carried out. Comparisons with other more commonly used genetic markers are needed in order to elucidate the implications of increased minisatellite mutations on populations.

Novel techniques for minisatellite mutation analysis at the single molecule level have a high sensitivity and speed for detecting both minisatellite length changes, and the rearrangement of variant repeat units along the tandem array. Application of SP-PCR will allow for the analysis of mutation rates and mechanisms of mutation in sperm for groups of human males who are highly exposed to genotoxins and will increase our understanding of the effect of chemicals on male germ-cells. In these studies we should compare both acutely and chronically exposed males. The mechanisms involved in generating spontaneous minisatellite mutation can be compared to chemically-induced mutations with
both MVR-PCR and SP-PCR and enhance our understanding of mutational spectra at
minisatellite loci.

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NOTE ADDED IN PROOF: A retraction was printed by Baker et al. [Nature, 390, p.
100, 1997] indicating that the mitochondrial cytochrome b mutation rates published for
the voles from the Chernobyl area [17] were incorrect as a result of miscoring of
mutations. The mutation rates in the voles near Chernobyl are higher than the control
population but this differences is not statistically significant with the sample size used.

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Table 3.1. Minisatellite mutation rates measured in offspring of humans inhabiting the radioactive contaminated area near Chernobyl and in families from the United Kingdom, and in herring gulls (*Larus argentatus*) colonizing an industrial urban location (Hamilton Harbour, Ontario, Canada) compared to three rural locations in the Great Lakes (pooled).

<table>
<thead>
<tr>
<th>Species (location)</th>
<th>No. of mutations</th>
<th>No. of bands scored</th>
<th>Mutation rate per band (minisatellite probes used)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Homo sapiens</em> (Chernobyl)*</td>
<td>49</td>
<td>1615</td>
<td>0.0303 (J33.15, MS32, CEB1)</td>
</tr>
<tr>
<td><em>Homo sapiens</em> (United Kingdom)*</td>
<td>23</td>
<td>1491</td>
<td>0.0154 (J33.15, MS32, CEB1)</td>
</tr>
<tr>
<td><em>Homo sapiens</em> (Chernobyl)†</td>
<td>136</td>
<td>6616</td>
<td>0.0206 (J33.15, J33.6, B6.1, CEB1, CEB15, CEB15, CEB25, CEB36, MS32)</td>
</tr>
<tr>
<td><em>Homo sapiens</em> (United Kingdom)†</td>
<td>56</td>
<td>5099</td>
<td>0.0110 (J33.15, J33.6, B6.1, CEB1, CEB15, CEB15, CEB25, CEB36, MS32)</td>
</tr>
<tr>
<td><em>Larus argentatus</em> (Hamilton)††</td>
<td>16</td>
<td>969</td>
<td>0.017 (J33.15, J33.6, PER)</td>
</tr>
<tr>
<td><em>Larus argentatus</em> (rural locations)††</td>
<td>12</td>
<td>2784</td>
<td>0.004 (J33.15, J33.6, PER)</td>
</tr>
</tbody>
</table>

* [33]
† [34]
†† [35]
Table 3.2. Mean band-sharing coefficients for pair-wise comparisons of DNA fingerprints from unrelated herring gull adults from Hamilton Harbour and the Bay of Fundy. Band-sharing coefficients were calculated with the formula: \( D = 2N_{AB} / (N_{A} + N_{B}) \) [60]. The Mantel test was used to compare band-sharing within and between colonies (NTSYS-pc Numerical taxonomy and multivariate analysis system).

<table>
<thead>
<tr>
<th>Location</th>
<th>n</th>
<th>J33.15</th>
<th>J33.6</th>
<th>PER</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hamilton Harbour</td>
<td>36</td>
<td>0.141 ± 0.102</td>
<td>0.149 ± 0.088</td>
<td>0.187 ± 0.122</td>
</tr>
<tr>
<td>Bay of Fundy (BoF)</td>
<td>37</td>
<td>0.201 ± 0.115</td>
<td>0.207 ± 0.178</td>
<td>0.178 ± 0.121</td>
</tr>
<tr>
<td>Between Hamilton and BoF</td>
<td>35</td>
<td>0.097 ± 0.048</td>
<td>0.166 ± 0.093</td>
<td>0.164 ± 0.116</td>
</tr>
</tbody>
</table>
Figure 3.1. DNA fingerprints from a family of herring gulls. The DNA was digested with *Hae* III and probed with J33.15. F and M designate the female and male parents. The nestlings are designated by n1-n3. A mutation in n3 is indicated with an arrow. This band can not be ascribed to a band in the lane of either parents.
CHAPTER 4
Genetic structure among Great Lakes and Eastern Canadian Herring Gull (*Larus argentatus*) populations

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ABSTRACT

Herring gulls (Larus argentatus) have been used as sentinel species for exposure to toxic chemicals since the 1960's. Control populations in these studies have never been genetically characterized to determine whether genetic differences might explain some of the effects seen in gulls at contaminated locations. We previously showed higher rates of germline minisatellite DNA mutation rates in herring gulls colonizing sites situated near steel industries in urban areas in the Great Lakes. We suggested that population substructuring among gull colonies analyzed could account for differences in the mutation rates observed as a result of surveying different minisatellite loci. Here we rule out this explanation by showing that genetic exchange among herring gull populations within the Great Lakes appears to be sufficient to ensure genetic homogeneity of these populations; in contrast, differences exist at the minisatellite loci scored between the gulls in the Great Lakes and the Maritimes. We suggest that control samples for future Great Lakes herring gull studies be selected from within the Great Lakes, and that potential genetic divergence from Maritime gulls should be investigated in more detail.

INDEX WORDS: minisatellite DNA, herring gull, Great Lakes, population genetics, genetic toxicology

INTRODUCTION

The herring gull (Larus argentatus) has been used as a sentinel species for chemical exposures and effects in the Great Lakes since the 1960's (Keith 1966, Ludwig and Tomoff 1966, Gilbertson and Hale 1974). This species is considered to be a valuable indicator species because it is a colonial-nesting, fish-eating predator present abundantly
throughout the Northern Hemisphere, and a year-round resident of the Great Lakes (Peakall and Fox 1987). Studies on Great Lakes herring gulls have revealed multiple effects correlated with exposure to pollution including: (a) the presence of persistent organic chemicals and heavy metals in Great Lakes herring gull eggs and tissues (Struger et al. 1987, Weseloh et al. 1990, Weseloh et al. 1994, Hebert et al. 1994, Weseloh et al. 1996, Koster et al. 1996,)(b) aberrant reproductive behaviour and reduced success (Keith 1966, Hickey and Anderson 1968, Gilman et al. 1977, Shugart 1980, Fitch and Shugart 1983, Conover 1984, Mineau et al. 1984) (c) changes in immune function (Grasman et al. 1996) and other biochemical changes (reviewed by Peakall and Fox 1987) and (d) an increased rate of germline mutations at minisatellite DNA regions in herring gull offspring hatched at colonies from industrial urban areas (Yauk and Quinn 1996, Yauk et al. in prep.).

Although the herring gull's behaviour, migratory movements and ecotoxicology have been studied extensively, little is known about the patterns of genetic variation in this species. An understanding of gene flow among the herring gull populations is critical for the proper choice of control colonies and for making appropriate between-colony comparisons for studies investigating the reproductive, biochemical, developmental, behavioral or genetic consequences of contaminant exposure. Information on the genetic makeup and variability of herring gull populations is essential before proper conclusions can be drawn on the effects, particularly genetic effects, of pollution exposure in herring gulls, and the use of herring gulls as a sentinel species across the Great Lakes.

In our previous research we investigated germline minisatellite DNA mutation rates in families of herring gulls from colonies that differed in the numbers, types and
distances from contaminant sources. We showed that minisatellite mutation rates were significantly higher at colonies situated near steel industries, compared to those in rural locations in the Great Lakes, and on the East Coast (Yauk and Quinn 1996). We suggested that differences in the herring gull mutation rates might be attributed to one or a combination of three causes: (1) differences in the genetic makeup of the colonies, (2) differences in the ages of parents sampled (as older parents may be more susceptible to mutation), and (3) differences in the exposure of the herring gulls to mutagenic agents. In this paper we explore the first of the three possible causes.

We have applied multi-locus DNA fingerprinting to investigate the minisatellite DNA of four herring gull colonies within the Great Lakes, and one colony on the East Coast of Canada, to determine whether differences exist in the genetic structure of the populations. Minisatellite DNAs are tandemly repeated arrays of nucleotides from 10 to 100 base pairs in length that are spread throughout the genome of eukaryotes (Jeffreys et al. 1985a). Minisatellite DNA sequences are the most variable regions found in the genome to date (Jeffreys et al. 1985a, b). Alleles in these non-coding regions vary in the number of repeats in the tandem array. The amount of band-sharing within and between populations is indicative of the extent of genetic exchange between the localities (Lynch 1991, Burke et al. 1991). In this study we show that gene flow between populations of herring gulls within the Great Lakes appears to be sufficient to ensure genetic homogeneity, but may be insufficient to prevent genetic differentiation from a population in the Maritimes.
METHODS

Sample Sites  Samples were collected from four sites; Gull Island in Presqu'ile Provincial Park (44° 00' N, 77° 43' W) and Hamilton Harbour (43° 15' N, 79° 51' W) are both situated on Lake Ontario. Chantry Island is offshore of Southampton, Ontario in Lake Huron (44° 29' N, 81° 23' W). The East Chicago site is on Lake Michigan in Indiana, U.S.A. (41°64' N, 87°45' W) on the industrial grounds of LTV Steel. The Maritime colony is Kent Island, a rural colony distant from the mainland in the Bay of Fundy on the Atlantic ocean (44° 34' N, 66° 45' W).

Sample Collection

Adult herring gulls were captured with drop traps set over their nests near the time of egg pipping in the spring of 1995. The birds were individually leg banded with numbered aluminum U.S. Fish and Wildlife Service bands. We collected 200 µl of blood from the brachial vein of each adult herring gull. Blood was stored in 1X lysis buffer (4.0 M urea/ 0.2 M NaCl / 0.1 M Tris-HCl, pH 8.0/ 0.5% n-lauryl sarcosine/ 0.1 M 1,2-cyclohexanediame) at 4°C.

DNA extraction

Approximately 25 µl of blood was digested twice with 83.3 units of proteinase K for 12 hours at 37°C. Samples were extracted twice with phenol/chloroform (70:30) and once with chloroform (Brock and White 1991) and then precipitated with 3.0 M sodium acetate and 95% ethanol. Concentrations of DNA were determined with a
minifluorometer (Hoefer, DyNA Quant 200) against a calf thymus DNA standard (Boehringer Mannheim).

**DNA fingerprinting**

A limitation to the use of DNA fingerprinting is the inability to compare between Southern blots. Therefore, we chose to compare each of the colonies to Hamilton Harbour, our primary study site in investigations on minisatellite mutation rates.

We generated 5 Southern blots to compare minisatellite band-sharing in herring gulls from Hamilton Harbour to each of the other colonies. We analyzed 18 randomly selected herring gull samples from Hamilton Harbour and 17 samples from Kent Island on 2 DNA fingerprinting blots. On three other blots we compared Hamilton Harbour herring gull samples (9 adults per blot) to: (1) 8 adults from Presqu’ile Park, (2) 9 adults from Chantry Island and (3) 9 adults from East Chicago.

For DNA fingerprinting, 3 ng of λ DNA size markers (Galbraith et al. 1991) were combined with 4.5 μg of *Hae*III-digested herring gull DNA and size fractionated by electrophoresis for 36-48 hours at 1.25-1.5 V/cm in 28 cm long, 0.8% agarose gels. Adenovirus DNA digested with *Bam*HI was run as a side lane marker. The gels were acid nicked with 0.25 M HCl for 15 minutes, denatured with 1.5 M NaCl/0.5 M NaOH for one hour and neutralized for 1 hour with 1.5 M NaCl/0.5M Tris base/ 1 M EDTA, pH 7.2. DNA was transferred to a polyvinylidene membrane (Immobilon N; Millipore) by Southern blotting and then air dried and baked at 80°C for 1-2 hours to fix DNA to the membranes. Blots were prehybridized at 64°C with a sodium orthophosphate prehybridization solution (Westneat et al. 1988) and sequentially probed with Jeffreys' 33.15, J33.6 (Jeffreys et al.
1985a) and finally λ. The J33.15 and J33.6 probes do not hybridize with λ DNA. A Sepharose spin column was used to separate unincorporated nucleotides from radioactively labeled probe. Blots were hybridized overnight and washed with 2X standard saline citrate/ 0.1% sodium dodecyl sulfate. Blots were autoradiographed at -70°C for short and long exposures. Probe DNA was stripped from blots between hybridizations with 0.4 M NaOH at 42°C and neutralized.

DNA fingerprinting analyses

We scored DNA fingerprint bands in the size range 3.5 to 23 kilobases. The minisatellite bands were identified across a blot by measuring the distance of migration from the nearest lambda in-lane size marker. Bands that co-migrated within 0.5 mm of each other were considered identical, unless they were less than half the intensity of their co-migrating counterparts. Pair-wise comparisons were made between each of the individuals on the same blots, and band-sharing coefficients (similarity indices or D-scores) were calculated with the formula $D = 2 \times \frac{N_{AB}}{N_A + N_B}$; where $N_{AB} =$ number of bands shared between individuals A and B; $N_A =$ the number of bands in individual A and $N_B =$ the number of bands in individual B (Wetton et al. 1987, Lynch, 1991). D-scores were calculated for both probes independently, as well as for the two probes pooled, after ensuring homogeneity of variance with a Bartlett's test. On each blot, D-scores within each of the colonies were compared to D-scores calculated between the colonies. The Mantel test (Rohlf, F.J., 1993; NTSYS-pc numerical taxonomy and multivariate analysis system, version 1.80) was used for statistical analysis of D-scores within and between the
colonies. With this test, a pseudo T value is calculated and then compared to 9999 permutations in which one matrix (a sample identification matrix) is randomized.

RESULTS

A high level of genetic variability is evident both within and between colonies (Table 1). Some variability exists in the D-scores calculated for band-sharing within Hamilton Harbour gulls across the different blots. The same 9 Hamilton Harbour individuals were run on the different DNA fingerprinting gels for the Chantry Island, East Chicago and Presqu'ile Park comparisons. A different set of 18 randomly selected herring gulls from Hamilton Harbour was compared to the Kent Island colony. Only small differences in the Hamilton Harbour D-scores were observed and may be attributed to slight differences in electrophoresis, hybridization or scoring. Comparisons were not made between blots. A sequential Bonferroni correction factor was used to account for multiple comparisons (Rice 1989).

The band-sharing coefficients from dyads within each colony were compared to band-sharing coefficients made up of an individual from Hamilton and an individual from another colony (between colony dyads) for both the independent and pooled probes (Table 1). D-scores within colonies of the Great Lakes were not significantly different from D-scores between the Great Lakes colonies and Hamilton Harbour (Table 2a). For all of the comparisons in the Great Lakes where within colony D-scores were greater than between colony D-scores, we replicated our matrices to effectively double the sample sizes in order to increase the power of our analyses to verify that our non-significant results were not just due to lower sample sizes. We then used the Mantel statistic to test
for differences between the sites again. We found no significant differences, suggesting that the lack of detectable structuring within the Great Lakes is probably not a result of inadequate sample size. These data suggest that herring gulls within the Great Lakes are as likely to share alleles with gulls from the same colony, as they are to share alleles with gulls from other Great Lakes colonies. Therefore, the gulls within the Great Lakes are not a statistically heterogeneous population. Conversely, using the same statistical criteria, D-scores for herring gulls within Kent Island were significantly higher than D-scores for Kent Island-Hamilton Harbour comparisons (between the colonies) with the probe Jeffreys' 33.15 and for the pooled probes (Table 2b) at the Bonferroni-corrected threshold p-value of 0.008. D-scores within Hamilton Harbour compared to D-scores between Hamilton and Kent Island were not significantly different (Table 2b). Furthermore, significantly higher band-sharing within Kent Island was detected compared to within Hamilton Harbour for both blots (pooled probes: matrix correlation coefficient = 0.346 and 0.358; p = 0.0001 and 0.0002 respectively) suggesting that Kent Island individuals are more related than Hamilton Harbour herring gulls. Kent Island herring gulls also have significantly higher band-sharing coefficients than the other Great Lakes colonies as well (Chantry; p = 0.0001; Presqu'ile; p = 0.0009; East Chicago; p = 0.0001). These data indicate that there may be different sets of minisatellite alleles scored within these two distinct populations, attributed primarily to differences detected with the probe J33.15.

We also examined gene flow among herring gulls in the Great Lakes with leg-banding data. In our investigations on the genetics of herring gulls in the Great Lakes, we trapped 18 adult herring gulls that were breeding at colonies in the Great Lakes and had previously been leg-banded as nestlings. Of the 18 breeding adults, 8 had left their natal
colonies to breed at other Great Lakes colonies (Table 3). Therefore, 44.4% of the previously leg-banded adults captured had dispersed to other colonies to breed.

**DISCUSSION**

Using multi-locus DNA fingerprinting, we found that herring gulls from colonies within the Great Lakes appear to be part of a large panmictic population. These large populations show a high level of genetic variability consistent with that expected for large outbred populations (Jeffreys *et al.* 1988). The population of herring gulls examined from Kent Island, on the East Coast of Canada, appears not to be part of the same panmictic population as the Great Lakes herring gulls.

Not all minisatellite loci undergo the same rate of mutation (Jeffreys *et al.* 1988). The most unstable loci found in humans are λMS1 and CEB1, with mutation rates of 5.2 and 6% per gamete respectively (Jeffreys *et al.* 1988, Vergnaud *et al.* 1991). If genetic differences among colonies resulted in only subsets of alleles from mutable loci being scored at certain colony sites (many alleles are too short to be scored on DNA fingerprinting gels), the resulting mutation rate would be inflated compared to other locations, potentially resulting in an over-estimation of induced mutation rate. It has also been discovered that one allele of the human minisatellite locus MS32 has a sequence variant upstream of the repeat units that acts to decrease the mutation rate (Monckton *et al.* 1994), and is present at high frequency in African populations compared to Caucasians and Japanese. Therefore, care must be taken in the proper selection of control populations for minisatellite studies because intrinsic differences may exist between minisatellite instabilities in different populations.
Here we have shown that there are no discernible genetic differences among herring gull colonies within the Great Lakes; therefore, genetic exchange among these herring gull colonies is likely sufficient to ensure genetic homogeneity of minisatellites among the Great Lakes colonies examined. These data are inconsistent with the hypothesis that minisatellite mutation rates are higher at Great Lakes steel sites compared to rural sites (Yauk and Quinn, 1996, Yauk et al. in prep.) as a result of genetic differences between these colonies. While we reject this hypothesis for the Great Lakes site, we can not reject this hypothesis for differences between the maritimes and the Great Lakes sites. Our data showed a significantly different subset of minisatellite alleles in Kent Island gulls compared to Hamilton Harbour. Furthermore, we show that Kent Island herring gulls are significantly more related within the colony than are the Hamilton gulls. We were unable to detect differences within Hamilton Harbour compared to between Hamilton and Kent Island. The low level of band-sharing within Hamilton Harbour makes it difficult to differentiate within colony D-scores from between colony D-scores. It has been suggested that the extremely high mutation rates at minisatellite loci, and the multi-locus nature of DNA fingerprinting, make the detection of inter-population differences against a background of high intra-population variability unlikely (Burke et al. 1991). Most studies applying minisatellite markers to questions on population genetics have focused on small populations, either historically or currently under the influence of restricted gene flow, and therefore exhibiting much lower levels of genetic variability (e.g., Gilbert et al. 1990, Degnan 1993, Patenaude et al. 1994). However, differences in outbred populations have been detected as well (Laughlin and Turner 1994). We have found that significant differences in outbred populations of herring gulls from the
Maritimes compared to the Great Lakes can be detected with multi-locus DNA fingerprinting.

Our results are consistent with leg band recovery data within the Great Lakes basin. Weseloh (1984) examined 14,901 leg bands recoveries within the Great Lakes basin from 1933 - 1980. His data showed that 99.4% of recovered bands were from herring gulls banded within the Great Lakes. Furthermore, of the 0.6% immigrants, 71% were less than three years of age (non-breeders). He therefore determined the maximum recruitment rate of immigrant gulls into the breeding population of the Great Lakes to be in the range of 0.1 - 0.2%. He concluded that in terms of immigration and recruitment, the adult herring gull population on the Great Lakes is essentially an isolated, closed system. Our genetic data provide support to Weseloh's (1984) conclusion on the isolation of the Great Lakes herring gulls from Eastern Canada. Although exchange between these colonies may occur rarely, it may not be sufficient to prevent genetic divergence between the populations.

The amount of exchange occurring among colonies within the Great Lakes is very relevant to the use of herring gulls as sentinel organisms for contaminant exposure within the Great Lakes. We used leg-banding data to examine dispersal of young of the year to breeding colonies in the Great Lakes. For many years prior to our studies, many herring gulls in Hamilton Harbour were banded as nestlings (pers. comm., Len Simser). Additionally, we trapped more intensively at Hamilton Harbour, as part of our large study on herring gull minisatellite DNA mutation rates, than at the other Great Lakes colonies. Consequently, we trapped a disproportionately high number of gulls banded as nestlings in Hamilton Harbour. Our field studies indicate that young of the year frequently disperse
from their natal colonies to breed in other locations in the Great Lakes. Of the captured
breeding adults which had been banded as nestlings (n = 18), 44.4% dispersed to other
colonies to breed. With such a high dispersal rate, it would be very surprising to find
significant genetic differences among colonies within the Great Lakes. Our genetic data
combined with our evidence for high dispersal rates among young herring gulls suggest
that the herring gulls of the Great Lakes are part of a large panmictic population.

To be conservative at this time, we suggest that control sites for herring gull
studies of the Great Lakes be selected from within the Great Lakes basin until genetic
differences are clarified. Furthermore, Kent Island herring gulls are inappropriate controls
for minisatellite studies of herring gulls in the Great Lakes.
REFERENCES


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Program under Environment Canada's Green Plan, the Wildlife Toxicology fund, a joint
effort by the World Wildlife fund and the Natural Sciences and Engineering Research
Council (of Canada) and the Canadian Wildlife Service of Environment Canada. Thanks
to the Ontario Government and the McMaster University Department of Biology for
financial support to CLY. We thank Dr. A.J. Jeffreys for providing the multilocus probes.
Thanks to Nathanial Wheelwright of Bodoin College, the Hamilton Harbour
Commissioners and LTV Steel for permission to work on their properties.
Table 4.1. Mean band-sharing coefficients and standard errors for pair-wise comparisons of DNA fingerprints from unrelated herring gull adults for 5 Southern blots. The Mantel test was used to compare band-sharing within and between colonies (NTSYS-pc numerical taxonomy and multivariate analysis system).

<table>
<thead>
<tr>
<th>LOCATION</th>
<th>n*</th>
<th>PROBE</th>
<th>Pooled</th>
<th>Probes</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>J33.15</td>
<td>J33.6</td>
<td></td>
</tr>
<tr>
<td>Blot 1.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hamilton (Ham)</td>
<td>9</td>
<td>0.16±0.25</td>
<td>0.12±0.14</td>
<td>0.13±0.10</td>
</tr>
<tr>
<td>Chantry Island (Chan)</td>
<td>9</td>
<td>0.07±0.20</td>
<td>0.09±0.09</td>
<td>0.08±0.11</td>
</tr>
<tr>
<td>Between Ham and Chan</td>
<td></td>
<td>0.12±0.13</td>
<td>0.12±0.13</td>
<td>0.12±0.09</td>
</tr>
<tr>
<td>Blot 2.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hamilton</td>
<td>9</td>
<td>0.16±0.30</td>
<td>0.12±0.24</td>
<td>0.15±0.10</td>
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<tr>
<td>East Chicago (EC)</td>
<td>8</td>
<td>0.12±0.09</td>
<td>0.13±0.06</td>
<td>0.15±0.16</td>
</tr>
<tr>
<td>Between Ham and EC</td>
<td></td>
<td>0.13±0.12</td>
<td>0.14±0.13</td>
<td>0.13±0.09</td>
</tr>
<tr>
<td>Blot 3.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hamilton</td>
<td>9</td>
<td>0.14±0.21</td>
<td>0.16±0.11</td>
<td>0.13±0.10</td>
</tr>
<tr>
<td>Presqu'ile Park (PSQ)</td>
<td>8</td>
<td>0.16±0.25</td>
<td>0.20±0.26</td>
<td>0.18±0.22</td>
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<tr>
<td>Between Ham and PSQ</td>
<td></td>
<td>0.13±0.13</td>
<td>0.16±0.12</td>
<td>0.16±0.10</td>
</tr>
<tr>
<td>Blot 4.</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hamilton</td>
<td>9</td>
<td>0.12±0.17</td>
<td>0.12±0.12</td>
<td>0.12±0.09</td>
</tr>
<tr>
<td>Kent Island (KI)</td>
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<td>0.17±0.09</td>
<td>0.17±0.15</td>
<td>0.17±0.06</td>
</tr>
<tr>
<td>Between Ham and KI</td>
<td></td>
<td>0.08±0.09</td>
<td>0.13±0.09</td>
<td>0.11±0.07</td>
</tr>
</tbody>
</table>
**Blot 5.**

<p>| | | | | |</p>
<table>
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<th></th>
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<tr>
<td>Hamilton</td>
<td>9</td>
<td>0.17±0.09</td>
<td>0.18±0.17</td>
<td>0.18±0.14</td>
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<tr>
<td>Kent Island</td>
<td>8</td>
<td>0.25±0.10</td>
<td>0.24±0.20</td>
<td>0.24±0.10</td>
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<tr>
<td>Between Ham and KI</td>
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<td>0.12±0.11</td>
<td>0.20±0.10</td>
<td>0.17±0.08</td>
</tr>
</tbody>
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* n = sample size
Table 4.2. Matrix correlation coefficients and associated p-values for the Mantel test for:

(a) 9 Hamilton Harbour herring gulls and 3 other Great Lakes colonies and (b) 2 independent blots comparing Hamilton Harbour and Kent Island herring gulls. The p-values are based on 9999 permutations. Significant differences are noted in bold.

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Probe</th>
<th>Difference</th>
<th>Matrix</th>
<th>p-value*</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>correlation**</td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>D-score*</td>
<td></td>
</tr>
</tbody>
</table>

(a) Hamilton Harbour and 3 other Great Lakes colonies

Blot 1

Chan vs B/W

<table>
<thead>
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<th>Probe</th>
<th>Difference</th>
<th>Matrix</th>
<th>p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>J33.15</td>
<td>-0.05</td>
<td>0.111</td>
<td>0.224</td>
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<tr>
<td>J33.6</td>
<td>-0.03</td>
<td>0.185</td>
<td>0.118</td>
</tr>
<tr>
<td>pooled</td>
<td>-0.04</td>
<td>0.188</td>
<td>0.0164</td>
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</tbody>
</table>

Ham vs B/W (Ham vs Chan)

<table>
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<th>Difference</th>
<th>Matrix</th>
<th>p-value*</th>
</tr>
</thead>
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<tr>
<td>J33.15</td>
<td>+0.04</td>
<td>0.004</td>
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<tr>
<td>J33.6</td>
<td>0.00</td>
<td>0.159</td>
<td>0.087</td>
</tr>
<tr>
<td>pooled</td>
<td>+0.01</td>
<td>0.073</td>
<td>0.193</td>
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Blot 2

EC vs B/W

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<th>Probe</th>
<th>Difference</th>
<th>Matrix</th>
<th>p-value*</th>
</tr>
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<tr>
<td>J33.15</td>
<td>-0.01</td>
<td>0.038</td>
<td>0.419</td>
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<tr>
<td>J33.6</td>
<td>-0.01</td>
<td>0.064</td>
<td>0.372</td>
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<tr>
<td>pooled</td>
<td>+0.02</td>
<td>0.029</td>
<td>0.442</td>
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Ham vs B/W (Ham vs EC)

<table>
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<th>Difference</th>
<th>Matrix</th>
<th>p-value*</th>
</tr>
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<tr>
<td>J33.15</td>
<td>+0.03</td>
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<tr>
<td>J33.6</td>
<td>-0.02</td>
<td>0.066</td>
<td>0.220</td>
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### Blot 3

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<th>Sample 1</th>
<th>Sample 2</th>
<th>Sample 3</th>
<th>Pooled 1</th>
<th>Pooled 2</th>
<th>Pooled 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSQ vs B/W</td>
<td>J33.15 +0.03</td>
<td>0.204</td>
<td>0.204</td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>J33.6 +0.04</td>
<td>0.187</td>
<td>0.075</td>
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<td></td>
</tr>
<tr>
<td>pooled</td>
<td>+0.02</td>
<td>0.129</td>
<td>0.054</td>
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<tr>
<td>Ham vs B/W (Ham vs PSQ)</td>
<td>J33.15 +0.01</td>
<td>0.142</td>
<td>0.152</td>
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</tr>
<tr>
<td></td>
<td>J33.6 0.00</td>
<td>0.038</td>
<td>0.380</td>
<td></td>
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</tr>
<tr>
<td>pooled</td>
<td>-0.03</td>
<td>0.194</td>
<td>0.019</td>
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</table>

### (b) Hamilton Harbour and Kent Island

#### Blot 4

<table>
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<tr>
<th>Test</th>
<th>Sample 1</th>
<th>Sample 2</th>
<th>Sample 3</th>
<th>Pooled 1</th>
<th>Pooled 2</th>
<th>Pooled 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>KI vs B/W</td>
<td>J33.15 +0.09</td>
<td>0.375</td>
<td>0.005</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>J33.6 +0.04</td>
<td>0.207</td>
<td>0.068</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>pooled</td>
<td>+0.06</td>
<td>0.385</td>
<td>0.000</td>
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<tr>
<td>Ham vs B/W</td>
<td>J33.15 +0.04</td>
<td>0.203</td>
<td>0.086</td>
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<td></td>
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<tr>
<td></td>
<td>J33.6 -0.01</td>
<td>0.065</td>
<td>0.369</td>
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<tr>
<td>pooled</td>
<td>+0.01</td>
<td>0.079</td>
<td>0.205</td>
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#### Blot 5

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<tr>
<th>Test</th>
<th>Sample 1</th>
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<th>Sample 3</th>
<th>Pooled 1</th>
<th>Pooled 2</th>
<th>Pooled 3</th>
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</thead>
<tbody>
<tr>
<td>KI vs B/W</td>
<td>J33.15 +0.13</td>
<td>0.474</td>
<td>0.006</td>
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<tr>
<td></td>
<td>J33.6 +0.04</td>
<td>0.151</td>
<td>0.165</td>
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<tr>
<td>pooled</td>
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<td>0.000</td>
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<tr>
<td>Ham vs B/W</td>
<td>J33.15 +0.05</td>
<td>0.194</td>
<td>0.133</td>
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<td></td>
<td>J33.6 -0.02</td>
<td>0.098</td>
<td>0.340</td>
<td></td>
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<tr>
<td>pooled</td>
<td>+0.01</td>
<td>0.233</td>
<td>0.479</td>
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</tbody>
</table>
*mean within colony D-score minus mean between colony D-score

** = normalized Mantel statistic Z

'probability (random Z<observed Z); Bonferroni equation used to correct for non-independent tests; new critical p value = 0.008
<table>
<thead>
<tr>
<th>Natal Colony</th>
<th>Breeding Colony</th>
<th>No. of adults captured</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hamilton, ON</td>
<td>Hamilton, ON</td>
<td>10</td>
</tr>
<tr>
<td>Sandusky, OH</td>
<td>Hamilton, ON</td>
<td>1</td>
</tr>
<tr>
<td>Sandusky, OH</td>
<td>East Chicago, IN</td>
<td>3</td>
</tr>
<tr>
<td>Sandusky, OH</td>
<td>Port Colborne, ON</td>
<td>1</td>
</tr>
<tr>
<td>South Bass Island, OH</td>
<td>Fighting Island, MI</td>
<td>1</td>
</tr>
<tr>
<td>7 km East of Chambers, WI</td>
<td>Port Colborne, ON</td>
<td>1</td>
</tr>
<tr>
<td>Erimley, MI</td>
<td>East Chicago, IN</td>
<td>1</td>
</tr>
</tbody>
</table>
Induced minisatellite germline mutations in herring gulls (*Larus argentatus*)
colonising locations near steel industries

C. L. Yauk, G.A. Fox*, B.E. McCarry and J.S. Quinn

Dept. of Biology, McMaster University, Hamilton, Ontario, Canada, L8S 4K1
*Canadian Wildlife Service, National Wildlife Research Centre, Hull, Quebec, K1A 0H3
Despite the genotoxic nature of many chemical contaminants released into our urban environment, little is known of their role in inducing germline mutations in populations inhabiting these environments. We used multilocus DNA fingerprinting to evaluate germline minisatellite mutations in herring gulls from breeding colonies near large steel mills with coking operations in cities, from colonies near cities that do not have steel mills and from rural locations within the Great Lakes. Gulls from sites near steel industries have significantly higher rates of germline minisatellite mutations than gulls from rural locations. Gulls from colonies near cities have mutation rates that are midway between rural and steel sites, and distance of the colony from steel industries was negatively correlated with germline minisatellite mutation rates. The results demonstrate significant risk for induced germline minisatellite mutations exists due to ambient levels of chemicals in urban environments near steel industries.

Recent investigations have utilised minisatellite DNA to measure induced germline mutations in populations sampled in their natural environment\textsuperscript{1,2,3}. Minisatellite DNA may provide the only marker currently available to measure statistically significant differences in germline mutations in populations exposed to complex mixtures of chemicals in situ\textsuperscript{3,4}. Minisatellite DNA is made up of DNA units from 10 to 100 bp in length that contain a common core sequence and are repeated in a tandem array\textsuperscript{5}. These loci are attractive targets for mutational surveys as they undergo a very high rate of mutation, are non-coding and therefore not subject to selective losses, and several loci can be examined simultaneously with DNA fingerprinting\textsuperscript{6}. We previously investigated germline
minisatellite mutation rates in a population of herring gulls inhabiting an industrialised urban location on the Great Lakes (Hamilton Harbour, Ontario, Canada), compared to two rural locations on the Great Lakes and one remote area in the Bay of Fundy. Gull nestlings from Hamilton inherited significantly more minisatellite mutations than gulls from rural locations. We suggested that mutation rates might be elevated at this site as a result of exposure to genotoxins in the Hamilton area. An alternative possibility is that mutation rates in Hamilton are elevated as a result of differences in the alleles scored in the populations, or differences in the age of the parents sampled.

In this study we investigate whether an elevated germline mutation rate reflects exposure to environmental contamination resulting from industrial sources. We examined multilocus DNA fingerprints of full families of herring gulls from 3 types of sites: (a) RURAL: rural colonies on the Great Lakes; (b) URBAN: one colony in a large city with no steel industry and one site near a small city with a nickel refinery; and (c) STEEL: colonies on the Great Lakes situated near large steel industries (Table 5.1).

The number of minisatellite mutations and the mutation rates per band scored for herring gull nestlings at each colony were determined (Table 5.2). A Nested ANOVA was used to test for differences in the number of mutations arising: (1) between the three main site classifications (rural, urban, steel) and between locations within each site; (2) between the three main sites, and years of study within each site. Significant differences between the sites were evident, with no significant heterogeneity between locations or years of study within sites (Table 5.3). Therefore, the mutation rates for different colonies within the site classifications were pooled. The minisatellite mutation rate was statistically
significantly higher at the steel site than the rural location (Table 5.2). The urban locations had a pooled mutation rate midway between rural and steel sites (Table 5.2).

Our lowest steel mutation rate was seen at Fighting Island near Detroit, Michigan, U.S.A. Because herring gulls here nest up to 9 km further from the coking ovens than the other steel sites (7 - 9 km from coking ovens) and in a predominantly upwind position relative to the industry (Table 5.1), exposure may be lower at this site. We subdivided herring gull colonies into 5 groups based on distance from steel industries and urbanisation. Classifications within the steel locations were based on distance of the sample site from the industrial coking ovens. The East Chicago colony was given a distance of 0 km as the colony is located on the industrial grounds. The Hamilton colony was subdivided into the 1.7-2.5 km group and the 4-9 km group based on nest location. A highly statistically significant positive correlation was found between herring gull mutation rate and proximity to steel industry (Figure 5.1, Kendall tau; Z=5.19; P<0.0001).

Mutation rates are not elevated due to genetic differences between the colonies. We found no differences in the subsets of minisatellite alleles across colonies within the Great Lakes (CLY and JSQ in prep.). Controlling for age in this investigation was not possible as herring gulls cannot be aged beyond their fourth year. However, it is very unlikely that we sampled an older subset of parents at each of the four steel sites. Additionally, nestlings hatched from a biased sample of known older parents (from Hamilton, Presqu’ile, Port Colborne, Chicago, Detroit and Chantry) showed minisatellite mutation rates that were similar to rates obtained at the rural locations. We examined 26 nestlings of older parents (>10 years of age; average lifespan is 12-15 years) and found a mutation rate of 0.0079 ± 0.0039, similar to typical rural colony rates.
Herring gulls are long-lived top predators that are year-round residents of the Great Lakes therefore chemical effects are not due to exposure to pollution in their migratory grounds. The gulls are predominantly fish eaters and may be exposed to contaminants through consumption of polluted fish. At the steel sites the gulls also are exposed to airborne contamination through coking process emissions as well vehicular emissions and other industries in the area. The predominant route of exposure to the herring gulls, and the mechanism and chemicals involved in inducing minisatellite mutations are currently unknown. Hundreds of chemicals are emitted from coking ovens, some of which show genotoxic activity; for example, polycyclic aromatic hydrocarbons are mutagens that are elevated in Hamilton Harbour air and sediment\(^7,8\). Importantly, the herring gulls colonising the city and steel sites inhabit urban areas where humans also live. Several investigations have shown somatic changes in the DNA of highly exposed humans employed in steel mills\(^9,10,11,12\) as well as increased rates of cancer\(^13\). Here, we show the risk of germ cell mutations may extend beyond those humans employed within the industry to organisms inhabiting the polluted urban areas near steel mills. The consequences of increased minisatellite mutations and their correlation to coding regions of the genome are currently unclear. A recent study on germline minisatellite mutation induced by radiation in mice showed that doubling dose estimates for paternal minisatellite mutation was 0.33 Gy, which is close to those obtained by traditional mutation scoring systems, including the specific locus test (Russell 7-locus test), previously the most efficient system for studying point mutations in mice\(^14\). This relationship suggests that minisatellite markers may provide a fast and sensitive method to screen for induced germ cell mutations that reflect damage in coding regions of the genome, pertinent to the health of the population.
Methods

Molecular analyses. Methods have previously been described in detail. Approximately 25 μl of herring gull blood was digested with proteinase k and phenol/chloroform extracted. DNA was digested to completion with Hae III and electrophoresed for 36 to 48 hours. Each sample was run with a lambda in-lane size marker to account for inter-lane variability. The gels were Southern blotted, prehybridized overnight with a sodium orthophosphate prehybridization solution at 64°C and probed with the multilocus probes Jeffreys 33.15, 33.6 and λ.

Fingerprint analysis. Mutations were identified as novel fragments in the lanes of nestlings which were not present in the lanes of either parents. We quantified the total number of novel bands arising in the lanes of the nestlings between 3.5 and 23 kb. DNA fingerprints were scored without knowledge of nest location. There were five cases where stem cell mutations were detected (i.e. two nestlings inheriting the same novel band; counted as one mutation) out of the 854 chicks examined. These mutations may have arisen very early in embryogenesis in parents, or result from stem cell heterogeneity, and may or may not have occurred at the colony location. In total, one stem cell mutation was detected in Hamilton, two in Sault Ste Marie and two in Port Colborne. These mutations were included in the analysis. The mutation rates for different localities are presented with the 95% confidence intervals derived from the Poisson distribution. A Nested Anova was used to test for differences in the number of mutations arising between site classifications,
between localities within sites and between years. Statistical procedures are described in Sokal and Rolf[8].

References


**Acknowledgments**

We would like to gratefully acknowledge Dr. Yuri Dubrova for performing the Nested ANOVA and the Kendall Tau. This research was supported by the National Cancer Institute of Canada with funds from the Canadian Cancer Society, the McMaster Eco-research Program for Hamilton Harbour through funds provided by the federal Tri-Council Eco-Research Program under Environment Canada's Green Plan, the Wildlife Toxicology fund, a joint effort by the World Wildlife fund and the Natural Sciences and Engineering Research Council (of Canada) and the Canadian Wildlife Service of Environment Canada. We thank Dr. A.J. Jeffreys for providing the multilocus probes.
Thanks to the Hamilton Harbour Commissioners, LTV Steel, the U.S. Army Corps of Engineers, BASF Corp at Fighting Island and Ed Newberry for access to their properties.

Correspondence and requests for materials should be addressed to J.S. Quinn (email: quinn@mcmaster.ca).
Table 5.1. Summary of sampling locations in the North American Great Lakes.

<table>
<thead>
<tr>
<th>Site</th>
<th>Location</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Rural sites:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chantry Island</td>
<td>44°29' N, 81°24' W</td>
<td>offshore, removed from point sources of pollutants</td>
</tr>
<tr>
<td>Presquile Park</td>
<td>44°00' N, 77°43' W</td>
<td>offshore, removed from point sources of pollutants</td>
</tr>
<tr>
<td>Double Island</td>
<td>46°10' N, 82°52' W</td>
<td>offshore, removed from point sources of pollutants</td>
</tr>
<tr>
<td>2. Urban sites:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Toronto</td>
<td>43°30' N, 79°23' W</td>
<td>population 4264 000</td>
</tr>
<tr>
<td>Port Colborne</td>
<td>42°54' N, 79°14' W</td>
<td>population 18 000, site of a nickel refinery</td>
</tr>
<tr>
<td>3. Steel sites:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Detroit</td>
<td>42°12' N, 83°07' W</td>
<td>population 1 028 000 and also near Windsor, Canada (population 279 000).</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7 to 9 km from coking ovens in a predominantly upwind position relative to the coking ovens</td>
</tr>
<tr>
<td></td>
<td></td>
<td>second largest steel manufacturing site of those sampled</td>
</tr>
</tbody>
</table>

93
<table>
<thead>
<tr>
<th>Location</th>
<th>Latitude, Longitude</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hamilton</td>
<td>43°15' N, 79°51' W</td>
<td>Population 624 000, 1.7 and 4 km from coke ovens, the 1.7 km site is predominantly downwind of ovens</td>
</tr>
<tr>
<td></td>
<td></td>
<td>largest concentration of steel industry on Canadian side of Great Lakes</td>
</tr>
<tr>
<td>Sault Ste Marie</td>
<td>46°29' N, 84°21' W</td>
<td>Population 84 000, 2.5 km from coke ovens, predominantly downwind position relative to industry</td>
</tr>
<tr>
<td></td>
<td></td>
<td>second largest concentration of steel industry on Canadian side of Great Lakes</td>
</tr>
<tr>
<td>East Chicago</td>
<td>41°64' N, 87°45' W</td>
<td>Population 34 000; surrounding populations: Gary, pop. 117 000, Chicago, pop. 2 784 000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>colony is situated on industrial grounds</td>
</tr>
<tr>
<td></td>
<td></td>
<td>largest concentration of steel industry on the Great Lakes</td>
</tr>
</tbody>
</table>
Table 5.2. Summary of minisatellite mutation data.

<table>
<thead>
<tr>
<th>Colony</th>
<th>No. of nestlings analyzed</th>
<th>No. of bands scored</th>
<th>Mean no. of bands per nestling</th>
<th>No. of Mutations*</th>
<th>Mutation rate per offspring band</th>
<th>95% CI</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Lower</td>
<td>Upper</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>1. Rural sites:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chantry Island</td>
<td>144</td>
<td>2699</td>
<td>18.7±0.3</td>
<td>19 (19)</td>
<td>0.0070</td>
<td>0.0042</td>
<td>0.0110</td>
</tr>
<tr>
<td>Presqu'ile Park</td>
<td>104</td>
<td>1979</td>
<td>19.0±0.5</td>
<td>9 (9)</td>
<td>0.0046</td>
<td>0.0021</td>
<td>0.0087</td>
</tr>
<tr>
<td>Double Island</td>
<td>35</td>
<td>576</td>
<td>16.5±0.6</td>
<td>4 (4)</td>
<td>0.0069</td>
<td>0.0018</td>
<td>0.0180</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>283</td>
<td>5254</td>
<td>18.6±0.3</td>
<td>32</td>
<td>0.0061</td>
<td>0.0042</td>
<td>0.0086</td>
</tr>
<tr>
<td><strong>2. City sites:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Toronto</td>
<td>81</td>
<td>1374</td>
<td>17.0±0.6</td>
<td>13 (12)</td>
<td>0.0095</td>
<td>0.0050</td>
<td>0.0162</td>
</tr>
<tr>
<td>Port Colborne</td>
<td>79</td>
<td>1677</td>
<td>21.2±0.6</td>
<td>14 (11)</td>
<td>0.0084</td>
<td>0.0046</td>
<td>0.0140</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>160</td>
<td>3051</td>
<td>19.1±0.5</td>
<td>27</td>
<td>0.0089</td>
<td>0.0058</td>
<td>0.0129</td>
</tr>
</tbody>
</table>
3. Steel sites:

<table>
<thead>
<tr>
<th>Location</th>
<th>N</th>
<th>M</th>
<th>p (±0.5)</th>
<th>c (±0.5)</th>
<th>μ</th>
<th>s.d.</th>
<th>%</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Detroit</td>
<td>44</td>
<td>713</td>
<td>16.2±0.6</td>
<td>7 (7)</td>
<td>0.0098</td>
<td>0.0039</td>
<td>0.0203</td>
<td></td>
</tr>
<tr>
<td>Hamilton</td>
<td>209</td>
<td>4051</td>
<td>19.4±0.4</td>
<td>49 (48)</td>
<td>0.0121</td>
<td>0.0090</td>
<td>0.0160</td>
<td></td>
</tr>
<tr>
<td>Sault Ste Marie</td>
<td>70</td>
<td>1118</td>
<td>16.0±0.5</td>
<td>14 (14)</td>
<td>0.0125</td>
<td>0.0089</td>
<td>0.0211</td>
<td></td>
</tr>
<tr>
<td>East Chicago</td>
<td>88</td>
<td>1636</td>
<td>18.6±0.6</td>
<td>23 (20)</td>
<td>0.0141</td>
<td>0.0068</td>
<td>0.0211</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>411</td>
<td>7518</td>
<td>18.3±0.3</td>
<td>93</td>
<td>0.0124</td>
<td>0.0100</td>
<td>0.0152</td>
<td>0.0004</td>
</tr>
</tbody>
</table>

*number of nestlings with mutations is indicated in brackets

† 95% confidence interval for the mutation rate derived from the Poisson distribution

‡ Probability of difference from the rural site (Fisher’s exact test, two-tailed)
<table>
<thead>
<tr>
<th>Source of variation</th>
<th>$F$</th>
<th>$df$</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Site classification and localities</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Between sites</td>
<td>12.86</td>
<td>2, 6</td>
<td>0.0068</td>
</tr>
<tr>
<td>Between localities within sites</td>
<td>0.51</td>
<td>6, 845</td>
<td>0.8033</td>
</tr>
<tr>
<td>Between sites and year of study</td>
<td>8.18</td>
<td>2, 9</td>
<td>0.0095</td>
</tr>
<tr>
<td>Between sites within years</td>
<td>0.80</td>
<td>9, 842</td>
<td>0.6178</td>
</tr>
<tr>
<td>Between years within sites</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 5.3: Results of Nested ANOVA.
Figure 5.1. Minisatellite mutation rates in herring gulls as a function of level of pollution, based on distance to steel industry. The p-values given over each group refer to the Fisher's Exact test (two tailed) difference between that group and the rural sites. The 'n' indicates the number of nestlings sampled within each group.
CHAPTER 6
DNA adducts in herring gulls (*Larus argentatus*) from a rural and industrial location using $^{32}$P-postlabelling

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and James S. Quinn*

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Abstract

Environmental pollutants showing genotoxic activity are elevated in industrialized urban areas. These pollutants include high molecular weight polycyclic aromatic hydrocarbons that can be metabolized to reactive intermediates that then form covalent bonds with DNA. Here, we use the $^{32}$P-postlabelling method to quantify the levels of adducts in DNA from blood cells of herring gulls (Larus argentatus) located in two colonies; an industrial urban site in Hamilton, Ontario, Canada, situated downwind of the highest concentration of steel industries in Canada, and Chantry Island, a rural location on Lake Huron. Our previous studies showed that gulls from Hamilton have significantly higher rates of germline minisatellite DNA mutations compared to Chantry Island herring gulls. While the exposure to airborne genotoxic compounds was significantly higher in Hamilton compared to Chantry Island, the gulls from the two colonies showed identical levels of total DNA adducts (approximately $9.6 \times 10^{-9}$ for both populations). Furthermore, there were no significant differences in the levels of various adducts (including the dG adduct of our benzo[a]pyrene diol epoxide standard), the patterns of adducts, or the number of individuals with detectable adducts. We suggest that DNA in erythrocytes of birds are not suitable targets for DNA-adduct analyses of biologically relevant exposures to PAH genotoxins. Furthermore, extrapolations to potential germ cell damage using erythrocyte DNA as a monitor of exposure is not possible.
Introduction

Anthropogenic chemicals are released from numerous sources into our urban and industrial environments. Many of the chemicals associated with fossil fuel combustion show genotoxic activity. The mutagenicity of ambient airborne particles and aquatic sediments in urban and industrial areas is reported to be largely associated with polycyclic aromatic compounds (DeMarini et al., 1994; DeFlora et al., 1990; Legzdins et al., 1993; Marvin et al., 1993). Polycyclic aromatic compounds (PACs) are generated from incomplete combustion of organic matter and figure prominently in risk assessments for occupational and environmental exposures (DeCaprio, 1997). Compounds like polycyclic aromatic hydrocarbons (PAH) exert their mutagenic effect through oxidative metabolism to reactive intermediates, which can then form DNA adducts and induce DNA damage if unrepaird.

Levels of DNA adducts are thought to provide a biologically relevant measure of exposure to genotoxic agents because they represent the integrated outcome of uptake, metabolism, distribution and reaction of the chemical with DNA (Nestmann et al., 1996). The measurement of the number of chemicals covalently bonded to DNA may therefore aid in the risk assessment for exposure to genotoxic chemicals found in the environment. Numerous studies have shown elevated levels of DNA adducts in populations of organisms highly exposed to bulky aromatic compounds in nature. For example, studies on fish inhabiting areas near highly contaminated sediments show higher levels of DNA adducts than fish from control populations (Dunn et al., 1987; Varanasi et al., 1989; Liu et al., 1991). Coke oven workers in steel industries have high occupational exposures to PACs and exhibit higher numbers of adducts in DNA from lymphocytes compared to
workers employed in low-exposure occupations (Øvrebø et al., 1992; Phillips et al., 1988; Rojas et al., 1995; Farmer et al., 1996). Additionally, residents inhabiting heavily polluted industrialized regions in Poland show significantly higher levels of DNA adducts than people from less polluted and rural areas (Whyatt et al., 1998; Perera et al., 1992).

Hamilton, Ontario, is the site of the greatest concentration of steel industries in Canada. Levels of PACs associated with inhalable air particulate (PM$_{10}$) collected downwind of the steel industries and in harbour sediments near these industries are known to be elevated relative to air and sediment samples collected further from the sources (Legzdins et al., 1993; Marvin et al., 1993; Murphy et al., 1990). We previously examined germline minisatellite mutation rates in populations of herring gulls at colonies in the Canadian Great Lakes that differ in their proximity to steel mills (Yauk and Quinn, 1996; Yauk et al., in prep.). We found a statistically significantly higher rate of germline mutations in herring gull colonies situated near large integrated steel industries that operate coking ovens compared to rural locations on the Great Lakes (Yauk et al., in prep.). Rates of germline minisatellite mutations in the Hamilton colony were nearly twice the rates found in rural locations in the Great Lakes, and 1.7 times higher than Chantry Island, the rural colony used in this study. We suggested that chemical contaminants present in the urban, industrial habitat of these herring gulls resulted in induced germline minisatellite mutagenesis. In this paper, we have used $^{32}$P-postlabelling to characterize the levels of DNA adducts in the erythrocytes of adult herring gulls from two of our sampling locations. It was our intention to determine whether this technique might be useful in evaluating relevant environmental exposures of natural bird populations to DNA-adduct forming chemicals. The two sampling locations were Chantry Island, Lake Huron, a rural
area well removed from point sources of pollutants, and Hamilton, an industrial urban area with two colonies located 1.7 and 4 km downwind of coking operations.

**Methods and Materials**

Sample sites: Chantry Island (44°29' N, 81°24' W) is situated offshore from Southampton, Ontario, Canada, on Lake Huron. This colony of herring gulls is well removed from highways, cities and industrial operations. Hamilton, Ontario (43°15' N, 79°51' W) is an industrialized urban location at the western tip of Lake Ontario. Hamilton has a large concentration of heavy industries. The two colonies in Hamilton are about 1.7 and 4 km from the coking operations of two integrated steel mills near two large cities (Hamilton, population 624 000 and Burlington, population 137 000). The gull colony is situated next to a heavily trafficked highway.

Sample collection: Adult herring gulls were captured with drop traps set over their nests late in the incubation period (late May and early June, in 1996). Blood (50 - 100 μl) was collected from the brachial vein of the birds. The blood was stored in cryotubes in liquid nitrogen in the field and later transferred to a -70°C freezer for long term storage.

Isolation of DNA: Approximately 25 μl of blood was dissolved in extraction buffer (0.2 M Tris, 25 mM EDTA, 0.1 M LiCl, pH 7.4) and digested with proteinase K. The blood digest was extracted once with phenol, once with phenol:chloroform:isoamyl alcohol (25:24:1) and once with chloroform:isoamyl alcohol (24:1). The extract was digested with RNase A and RNase T1, proteinase K digested and re-extracted with
chloroform:isoamyl alcohol. DNA was quantified with a minifluorometer (Hoefer, DyNA Quant 200) against a calf thymus standard.

$^{32}$P-postlabelling: Twenty micrograms of DNA was digested with micrococal nuclease (Sigma) and spleen phosphodiesterase (Boehringer Mannheim) (MN/SPD). Calf thymus DNA was reacted with several concentrations of anti-BPDE ((±)-anti-7R,8S-dihydroxy-9R,10R-7,8,9,10-tetrahydro-BP, BP diolepoxide, BPDE) (Chemsyn, Lenexa, KS, USA) as described in Baan et al. (1997). This standard was treated as above from the MN/SPD digest on, and was used in our experimental procedure to account for inter-experiment variability. The nucleotide digest was butanol enhanced twice and was back-extracted three times with water and vacufuged (Gupta, 1985). The mixture of normal nucleotides and DNA adducts was labeled with $\gamma^{32}$P-dATP (6000 Ci/mmol, Mandel Scientific) with T4 polynucleotide kinase (Boehringer, Mannheim, Germany). Excess $^{32}$P-dATP was degraded with potato apyrase (Sigma).

Thin layer chromatography: Labelled nucleotide digests were run on poly(ethylenimine) (PEI)-cellulose TLC sheets prepared in our laboratory. Total nucleotide concentration in digests was determined by chromatography in 0.3 M ammonium sulphate/10 mM sodium phosphate. Adducts were separated using two dimensional chromatography using the following solvents: D1, 1.0 M sodium phosphate, pH 6.8; D2, 2.5 M ammonium formate, pH 3.5; D3, 3.0 M lithium formate, 8.5 M urea, pH 3.5; D4, 0.8 M lithium chloride, 0.5 M Tris-HCl, 7.0 M urea, pH 8.0; D5, 1.0 M sodium phosphate, pH 6.8 (Gupta et al., 1982).
DNA adduct analyses: Normal nucleotides concentrations were determined using a phosphor-imaging system (Molecular Dynamics, model 425B). DNA adduct TLC plates were placed on phosphor-image screens for 16-24 hours. Calibration strips were simultaneously phosphor-imaged to generate a conversion factor for density to dpm.

Distinct spots were identified by comparison to a sample of authentic BPDE adducts which was run with each batch of TLC plates; in no cases were diagonal radioactive zones evident. A distinct pattern of spotting was evident in the herring gull TLC's. The spots were named BaP (because of migration to the same location as the major spot in our calf thymus benzo[a]pyrene-diol-epoxide standard) and arbitrarily A - G (Figure 6.1). No effort was made to unequivocally characterize “BaP” in gull DNA digests with authentic BPDE adducts. The frequency of occurrence of each spot, the number of adducts for each of the spots, as well as the total number of adducts were determined. The number of adducts for each spot and the pattern of spots for Chantry Island and Hamilton Harbour herring gulls were compared and evaluated using the Mann-Whitney U test and Chi-squared tests.

Air Sampling: An Anderson PM₁₀ hi-vol air sampler (General Metal Works, Village of Cleves, OH) was used to collect 35 (24-hour) samples of inhalable airborne particulate near the Hamilton herring gull colonies, and 2 samples from a provincial park located near Chantry Island (MacGregor Point). Samples were collected in May and in June in Hamilton, and in June at MacGregor Point. Each sample collected approximately 1630 m³
of air over a 24 hour period on 8 X 10 inch Teflon-coated glass fibre filters (Pallflex TX40M120WW).

Soxhlet Extraction: Methods are modifications of those described in Legzdins et al. (1995). Briefly, air filters were extracted using a Soxhlet apparatus with 180 ml of dichloromethane for 24 hours. The dichloromethane and methanol extract was reduced in volume about 2-3 ml, applied to an alumina Sep-Pak (Water, Milford, MA) and the Sep-Pak eluted with an additional 5 ml of dichloromethane. The resulting non-polar PAC fraction was reduced in volume to about 100 µl and chromatographed on a Sephadex LH20 column (mobile phase: hexane/methanol/ dichloromethane, 6:4:3, v/v). Naphthalene was analyzed prior to the separation. Compounds (aliphatic and benzene derivatives) eluting before the naphthalene standard were discarded. Components eluting after the start of the naphthalene peak were collected as the non-polar aromatic fraction.

GC-MS Analyses: Analyses were performed on a Hewlett-Packard Model 5890 Series II gas chromatograph equipped with electronic pressure control, an on-column injector, a mass selective detector (Hewlett-Packard Model 5971A MSD) and an autosampler (Hewlett-Packard Model 7673). The GC-MS operating conditions were as follows: transfer line temperature: 300°C; helium carrier gas velocity: 30 cm/sec. The following temperature program was used for PAC analyses: 90° to 300°C at 2.5°C/min then hold for 10 min at 300°C. A DB-17ht column (30 m X 0.25 mm i.d. 0.25 µm stationary phase film, J&W Scientific, Folsom, CA) was used for all analyses. A recovery standard consisting of phenanthrene-d_{10}, benz(a)anthracene-d_{12} and dibenz(a,h)anthracene-d_{14} was
added to the Soxhlet apparatus prior to extraction. A calibration standard containing pyrene-d_{10} and perylene-d_{12} was added prior to GC-MS analysis. Column and system performances were checked daily by injection of a PAC standard consisting of 45 standard compounds.

**Results**

A total of 19 adult herring gulls from Chantry Island and 19 from Hamilton were examined. The total levels of DNA adducts were similar for the two sites. The mean numbers of detectable adducts were: $9.7 \pm 9.1 \times 10^{-9}$ and $9.4 \pm 9.1 \times 10^{-9}$ for Chantry Island and Hamilton gulls respectively. The levels of adducts detected ranged from 0.01 to $35 \times 10^{-9}$. A sequential Bonferroni correction was used to adjust for non-independent tests (Rice, 1989). No statistically significant differences in adduct levels were found for specific spots within the TLCs. There was no evident variation in the frequencies of specific spots between the two colonies, nor were differences evident in the patterns of specific DNA adducts (Table 6.1) at the Bonferroni corrected p-value of 0.006. In total, 13 individuals from Chantry Island (68%), and 15 from Hamilton (79%) had detectable levels of DNA adducts.

The levels of total PAH in inhalable air particulate collected on filters near the Hamilton colony ranged from 0.8 to 255 ng/m$^3$ and depended on wind directions and wind speeds; the average value for the 35 days was 55 ng/m$^3$. Only 2 filters were collected near Chantry Island, and their values were both less than 1 ng/m$^3$. The mean level of PAH exposure at the Hamilton colony is more than 50 times the level at Chantry Island, indicating that the birds in Hamilton are exposed to greater ambient levels than at the rural location.
Discussion

DNA adduct analysis of herring gull erythrocytes has never been reported previously. Herring gull erythrocytes did not reveal differences in the DNA-binding of chemical mixtures between our urban/industrial and rural locations. All of the adducts detected in herring gulls from Hamilton were also present at the rural location, and at similar levels, indicating that pollution-related aromatic, hydrophobic or aromatic and hydrophobic DNA adducts were not present in DNA from this source. Surprisingly, a major adduct that co-migrated with our benzo[a]pyrene diol epoxide standard was present in herring gull samples from our rural location. While co-migration can not be considered unequivocal evidence that two adduct spots are identical chemical composition, the fact that this spot appeared in both populations presents an interesting problem. The rural herring gulls are removed from cities and industries and to our knowledge not foraging near any known point sources of pollutants. It is known that certain toxins are distributed throughout the Great Lakes and accumulate in herring gulls (Stow, 1995; Weseloh et al., 1990). Thus, every species in the Great Lakes is exposed to pollutants. However, the majority of these compounds are pesticides or polychlorinated biphenyls and are not thought to play major roles in adduct-formation compared to PAH. Compounds like PAH are quickly metabolized in vertebrates and do not bio-accumulate. We are not aware of any PAC-contaminated sediments or point sources of PAC near the herring gull colony at Chantry Island; however, herring gulls can forage up to 30 kilometers away from their colony location (Morris and Black, 1980).
Kurulec et al. (1989) encountered similar results in their study of various species of fish foraging in areas with highly contaminated sediments compared to unpolluted areas. The authors used $^{32}$P-postlabelling to determine the level of DNA modifications in liver DNA of 5 species of fish. They suggested that the vast majority of DNA adducts detected in fish are caused by 'natural' environmental factors rather than pollution. Additionally, they suggested the adducts may arise naturally as a result of unknown endogenous factors, including hormones. Schilderman et al., (1997) were not able to detect higher levels of PAH-related DNA adducts in the liver, kidney and lungs of wild pigeons between high and low traffic areas in Amsterdam, despite differences in the ambient air PAH concentrations. Similarly, some studies examining DNA adduct levels in highly exposed humans (e.g., smokers) have failed to show higher levels of DNA adducts in lymphocytes compared to control populations (Jahnke et al., 1990; Phillips et al., 1988). The authors suggest differences might result from individual variation in metabolism and/or DNA repair.

The levels of DNA adducts measured in this study are at the lower end of normal adduct levels detected in circulating lymphocytes of humans from environmentally polluted areas using $^{32}$P-postlabelling (herring gulls; mean is less than $1 \times 10^{-8}$ for both sites, with a range of $0.001$ to $3.6 \times 10^{-8}$; Perera et al., 1992; humans; mean for control population in summer = $2.9 \times 10^{-8}$, mean for exposed population in winter $5.5 \times 10^{-8}$; range of $0.3$ to $18.0 \times 10^{-8}$). Several reasons might explain the low DNA adduct levels found in herring gulls from Hamilton despite differences in exposure: (1) birds have nucleated erythrocytes which may provide a dilution effect (i.e., a lot of target DNA) for DNA binding chemicals in blood, (2) higher numbers of histones in bird blood may protect DNA by preventing
binding from occurring (Stevens, 1996), (3) higher rates of metabolism may result in the elimination of DNA binding chemicals.

The reasons listed above suggest that nucleated erythrocytes may not be appropriate target cells for DNA adduct analyses in general, and more specifically, probably are not relevant to damage which might be occurring in germ cells. It has been shown that levels of DNA adducts in circulating lymphocytes in humans and mice are detected at lower levels than in other organs, like lung and heart, and may not reflect damage accumulation in target organs (Lewtas et al., 1993; Binkova et al., 1994). In pigeons from highly polluted areas of Amsterdam (Schilderman et al., 1997), much higher levels of PAH-related DNA adducts were found in kidney and liver compared to lung cells. DNA adduct levels in erythrocytes were not determined in the Schilderman et al. study. Therefore, our results do not necessarily indicate that levels of DNA damage in other tissues or cells, including germ cells, are not higher in Hamilton.

The results of this study show that DNA adduct levels in erythrocytes of gulls are not relevant biomarkers of exposure to PAH. In our previous studies we showed that germline minisatellite mutation rates are elevated in the Hamilton herring gulls compared to our rural location (Yauk and Quinn, 1996; Yauk et al., in prep.). These studies specifically measured germ cell changes, and therefore can not be related to levels of adducts found in erythrocytes. It would be worthwhile to determine the DNA adduct levels in specific target tissues in order to clarify overall exposure, as well as predominant routes of exposure to PACs. Our goal in this study was to clarify overall exposure to PAC's in a non-invasive manner in the herring gull populations. We suggest that future investigations should determine DNA adduct levels in lung, liver and gonads, in order to
evaluate whether DNA adduct forming chemicals might be important in the generation of minisatellite mutations.

Lastly, the mechanisms and chemicals involved in generating minisatellite mutations are unclear, and polycyclic aromatic compounds may, or may not, be important in their manifestation. Exposure to diesel exhaust extracts has been shown to induce germ cell minisatellite mutations in mice (Hedenskog et al., 1997). Presumably, the PAC in these extracts were responsible for these mutations. However, in the same study it was shown that mixtures of polychlorinated biphenyls (PCBs) were more potent inducers of minisatellite mutations than diesel exhaust extracts alone. The genotoxicity of PCBs is poorly understood and Hedenskog et al. suggest that the recombinogenic activity of PCBs (Butterworth et al., 1995; Aubrecht et al., 1995) might be important in inducing minisatellite mutations despite the lack of significant differences in mutation rates between pre- and post-meiotic sperm cells. Dubrova et al. (1998) showed that exposure to acute ionizing radiation in mice results in minisatellite changes in pre-meiotic, but not post-meiotic, stages of spermatogenesis, indicating that the mechanism is recombination based. Therefore, chemicals present in the Hamilton environment that may possess recombinogenic activity should be evaluated in more detail.

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Hedenskog, M., Sjögren, M., Cederberg, H., Ranug, U., (1997) Induction of germline-length mutations at the minisatellites PC-1 and PC-2 in male mice exposed to


Perera, F.P., Hemminki, K., Gryzbowska, E., Motykiewicz, G., Michalska, J., Santella, R.M., Young, T., Dickey, C., Brandt-Rauf, P., DeVivo, I., Blaner, W., Tsai, W., Chorazy,


Yauk, C.L., Quinn, J.S., Fox, G.A. (in prep.) Induced minisatellite germline mutations in herring gulls (*Larus argentatus*) colonising areas near steel industries.
Table 6.1. DNA adducts quantified in erythrocytes of adult herring gulls from Hamilton and Chantry Island, and frequency of specific adduct spots. A two-tailed Mann Whitney U score and p-value is given for comparison of DNA adduct levels between Hamilton and Chantry Island, and a $\chi^2$ test was used to compare the frequency of specific DNA adduct spots between the two sites. P-values near 0.05 are highlighted in bold print.

<table>
<thead>
<tr>
<th>Spot</th>
<th>Chantry Island</th>
<th>Hamilton</th>
<th>U</th>
<th>p</th>
<th>$\chi^2$</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=19</td>
<td>n=19</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BaP</td>
<td>3.3 ± 3.0</td>
<td>2.8 ± 2.8</td>
<td>70</td>
<td>0.517</td>
<td>1.15</td>
<td>0.290</td>
</tr>
<tr>
<td>A</td>
<td>4.4 ± 4.3</td>
<td>3.1 ± 3.7</td>
<td>51</td>
<td>0.142</td>
<td>0.12</td>
<td>0.732</td>
</tr>
<tr>
<td>B</td>
<td>0.9 ± 0.7</td>
<td>1.0 ± 1.1</td>
<td>50</td>
<td>0.776</td>
<td>0.96</td>
<td>0.328</td>
</tr>
<tr>
<td>C</td>
<td>2.9 ± 2.3</td>
<td>0.8 ± 0.1</td>
<td>1</td>
<td>0.165</td>
<td>0.93</td>
<td>0.335</td>
</tr>
<tr>
<td>D</td>
<td>1.3 ± 1.0</td>
<td>1.6 ± 1.8</td>
<td>16</td>
<td>0.572</td>
<td>4.07</td>
<td>0.044</td>
</tr>
<tr>
<td>E</td>
<td>0.8 ± 0.7</td>
<td>0.8 ± 0.9</td>
<td>8</td>
<td>0.796</td>
<td>1.31</td>
<td>0.252</td>
</tr>
<tr>
<td>F</td>
<td>0.3 ± 0.1</td>
<td>1.2 ± 1.9</td>
<td>14</td>
<td>0.396</td>
<td>0.96</td>
<td>0.328</td>
</tr>
<tr>
<td>G</td>
<td>0.3 ± 0.4</td>
<td>1.2 ± 2.2</td>
<td>27</td>
<td>0.955</td>
<td>5.22</td>
<td>0.022</td>
</tr>
<tr>
<td>Mean</td>
<td>9.7 ± 9.1</td>
<td>13†</td>
<td>9.4 ± 9.1</td>
<td>15</td>
<td>64</td>
<td>0.337</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>Total adducts</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* the number of times spots occurred in the 19 herring gulls analyzed
† indicates the number of herring gulls with detectable DNA adducts
Figure 6.1. DNA adduct profiles from a BPDE calf thymus standard (dG-BPDE spot is labelled) and herring gulls: (a) a heavily adducted sample from Hamilton and (b) a more typical TLC of herring gulls from Chantry Island and Hamilton.
CHAPTER 7
SYNTHESIS AND RECOMMENDATIONS FOR FUTURE RESEARCH
7.1 Synthesis

Screening for germline mutations in natural populations exposed to ambient concentrations of chemicals is very difficult because of the rarity of induced heritable mutations. I have successfully demonstrated the application of minisatellite DNA mutation rate as a biomarker for induced germline mutations in a free-living vertebrate species, the herring gull, from populations exposed to ambient levels of urban and industrial contamination. This is the first identification of a biomarker to that is able to measure statistically significant differences in germline mutations occurring in situ as a result of exposure to non-radioactive chemicals. Furthermore, this study is the first to show a statistically significant correlation between germline mutations (important at the population level because they are heritable) and proximity to steel industries. Lastly, it demonstrates that germ cell mutagens may be emitted from steel processes into the urban environment, where humans also live. The culmination of 6 years of collecting herring gull samples and running DNA fingerprints is presented in Chapter 5.

The alternative hypotheses for differences in mutation rates among herring gull colonies include: (1) genetic differences in the populations or (2) differences in the ages of parents sampled, as older parents might be more susceptible to germ cell mutations.

Potential genetic differences between the herring gull colonies was addressed in detail in Chapter 4. Differences in the subsets of minisatellite loci scored were not evident between herring gull colonies within the Great Lakes. However, statistically significant differences in band-sharing were evident between the Maritime control colony and Hamilton Harbour, suggesting that differences in the minisatellites scored might exist for these two locations. Therefore, based on the possibility of allelic differences in the
minisatellites scored between the East Coast and the Great Lakes, the rural colonies selected for comparisons to industrial and urban colonies, in Chapter 5, were all from Great Lakes populations. Genetic differences in Great Lakes populations do not explain differences in the observed minisatellite mutation rates.

The age hypothesis is difficult to address in herring gulls as they can not be aged beyond their fourth year. As a result, it is not possible to determine the age of the parents in order to evaluate whether older parents were sampled from sites that showed higher minisatellite mutation rates. Given the sample sizes, it is unlikely that subsets of older parents were sampled at each of the four steel sites. In order to address this hypothesis in more detail, I determined minisatellite mutation rates from a known aged sample of older parents from several colonies, including industrial sites. In total, I evaluated mutation rates in 26 nestlings, from 11 sets of older parents (>10 years of age; average lifespan is 12-15 years), and found a mutation rate of 0.0079 ± 0.0039 per minisatellite band scored, similar to typical rural colony rates (range of 0.0046 - 0.0070). The ages of these parents were determined from leg-banding data. Older parents do not appear to have increased mutation rates and therefore this hypothesis can not explain the differences observed in the minisatellite mutation rates. Furthermore, there is no reason to suspect that we have sampled different age groups of parents among the Great Lakes colonies.

The data in this thesis are consistent with the hypothesis that germline minisatellite mutation rates are elevated at the steel sites as a result of the elevated levels of chemical mutagens present in these environments. This hypothesis is strengthened by the correlation between distance to coking ovens and induced minisatellite mutation rates (Chapter 4). The colony showing the highest rate of mutation is located on the grounds of
LTV Steel in East Chicago, where the highest concentration of steel industry on the Great Lakes exists. The herring gull nestlings exhibiting the lowest rates of mutation in an environment near coke ovens were sampled from Fighting Island; this colony is located up to 9 km directly upwind of the industry at this location.

The herring gulls at steel sites may be exposed to contaminants through their aquatic diets, or through inhalation of airborne particulate and chemicals. However, given the correlation between distance of the herring gull colonies relative to the coking ovens of steel industries, and the position of the Fighting Island colony relative to prevailing winds, air contamination seems to be the most plausible route of exposure. The Fighting Island colony is downstream of the steel industry, and the collected sediments near the colony are known to be contaminated with chemical mutagens (Fallon and Horvath, 1985; Kaiser et al., 1985; Furlong et al., 1988). Bullhead (*Ameiurus nebulosus*) populations in the Detroit River show a high incidence of external lesions, like tumours and other deformities associated with chemical contaminant exposures (Leadley et al., 1998). Therefore, the gulls at the Fighting Island colony may be ingesting contaminants through their aquatic diets. However, because they are upwind and further from the coking ovens, the gulls at Fighting Island are probably exposed to lower levels of airborne contaminants (I did not evaluate the levels of airborne contaminants at this site) than at the other steel sites (McCary, personal communication). Therefore, although the routes of exposure to the herring gulls are unclear at this time, it seems reasonable to postulate that the predominant route of exposure may be through airborne contamination. Routes of exposure to the herring gulls should be evaluated in more detail, in order to clarify the sources of risk, and what other populations are potentially exposed.
To estimate biologically relevant exposure, I determined the levels of DNA adducts found in herring gulls from Hamilton, Ontario, compared to gulls from one rural location, Chantry Island, Lake Huron. The levels and patterns of DNA adducts in herring gull erythrocytes from the two sites were essentially identical. I suggested that erythrocytes may not be appropriate target tissues for DNA adduct analyses (Chapter 6), and probably do not correlate with germ cell lesions. Also, because the mechanism resulting in induced minisatellite mutations appears to be indirect and recombination-based (Dubrova et al., 1998), chemicals that form DNA-adducts may not be the principal mutagens involved in minisatellite mutagenesis. Compounds that show recombinogenic activity should be examined in more detail.

7.2 Recommendations for future research

This research has answered a number of questions but has raised other important questions: (1) what are the particulate materials and/or chemical mutagens emitted from steel mills that are responsible for the observed increased mutation rates, (2) what is the mechanism that results in induced minisatellite mutation, (3) what is the predominant route of exposure (4) what is the implication of increased minisatellite mutation rate to the health of populations, including humans who inhabit the same areas as herring gulls.

The primary mutagens that result in induced minisatellite germline mutations are currently unknown. Studies on germline minisatellite mutations in mice show that the mechanism of mutation induction is indirect. Because the number of strand breaks predicted based on exposure can not explain the increased rate of minisatellite mutation, it has been suggested that damage to other areas of the genome, or other molecules, may be
important in generating mutations (Dubrova et al., 1997; Dubrova et al., 1998). More specifically, it has been suggested that chemicals that induce recombination might be important in this process. In a study by Hedenskog et al. (1997), it was shown that PCBs are more potent inducers of minisatellite germline mutations in mice than diesel exhaust. The primary mutagens in diesel exhaust are polycyclic aromatic hydrocarbons (PAH). PAH are potent mutagens and carcinogens in laboratory assays. In contrast, the genotoxicity of PCBs is poorly understood, and much less important compared to PAH in standard laboratory assays. Hedenskog et al. suggest that the recombinogenic activity of PCBs (Butterworth et al., 1995; Aubrecht et al., 1995) might be important in inducing minisatellite mutations. Similarly, Nakagama et al. (1997) showed an increase in minisatellite mutations after exposure to okadaic acid (OA) in vitro. Because OA does not appear to interact directly with DNA, Nakagama et al. suggest that perturbation of the phosphorylation status of proteins (OA is an inhibitor of serine/threonine phosphatases) could be a contributor to induced minisatellite instability. They suggest that alterations in the phosphorylation of proteins that bind to minisatellite regions may lead to changes in recombination machinery and consequently result in minisatellite mutations.

Therefore, future investigations should focus on the determination of the chemicals involved in the generation of minisatellite mutations. Studies investigating populations exposed to high levels of chemicals, like PCBs, should be undertaken to clarify the mutagenic agents responsible and other potential sources of minisatellite mutagens. Laboratory studies on mice exposed to single chemical agents, including PAH, PCBs, dioxins, metals and other chemicals found at high levels in environmental media, may clarify the potency of groups of chemical classes, and the dose response relationships,
in induced minisatellite mutations. Application of single locus minisatellite markers can differentiate between maternal and paternal contributions to germline mutations.

Additionally, with the use of single locus markers, the DNA sequence of de novo mutations can be determined and may provide information about the mechanisms involved in generating minisatellite mutations.

Route of exposure to the herring gulls should also be investigated in more detail. The distinction of inhaled versus ingested contamination will clarify the groups of organisms that are most at risk for induced minisatellite germline mutagenesis. Studies investigating the minisatellite mutation rates found in groups of humans exposed to high levels of mutagens occupationally should be undertaken, especially in coke oven workers.

Finally, the implications of induced minisatellite mutations on the health and reproduction of populations should be studied. It has been shown that estimates of doubling dose for radiation exposure in mice using minisatellite markers correspond to estimates for other marker systems, suggesting that increased minisatellite mutations may correlate to DNA damage in other regions of the genome (Dubrova et al., 1998). Additionally, the functional role of minisatellite DNA regions in the genome is unclear. Although the majority of minisatellites are thought to be in non-coding regions of the genome, several minisatellite markers are associated with important disease-related genes in humans (see Chapter 3). Therefore, it is unclear how minisatellite mutations affect overall health of an organism.
References


Appendix 1

Glossary of frequently used acronyms:

BaP: benzo[a]pyrene
BPDE: benzo[a]pyrene-diol-epoxide
B/W: between colonies
CA: chromosome aberration
Chan.: Chantry Island
DNA: deoxyribonucleic acid
DSB: double strand break
EC: East Chicago
Ham.: Hamilton Harbour

*hp*rt: Hypoxanthine Phosphoribosyl Transferase gene

J33.15: minisatellite probeJeffreys’ 33.15
J33.6: minisatellite probeJeffreys’ 33.6

MN: micronuclei

OA: okadaic acid

PAC: polycyclic aromatic compounds

PAH: polycyclic aromatic hydrocarbon

PCB: polychlorinated biphenyls

PCR: polymerase chain reaction

SCE: sister chromatid exchange

SP-PCR: small pool PCR
SSB: single strand break

TLC: thin layer chromatography