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GERMLINE MUTATIONS IN SENTINEL MICE

GERMLINE MUTATIONS AT EXPANDED SIMPLE TANDEM REPEAT DNA
LOCI IN SENTINEL MICE

By

CHRISTOPHER MICHAEL SOMERS

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Studies in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy

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AUTHOR: Christopher Michael Somers, B.Sc. (York University), M.Sc. (Brock
University)

SUPERVISOR: Professor James S. Quinn

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THESIS ABSTRACT

Exposure to environmental contaminants that can cause genetic damage may pose a risk of induced heritable mutations in wildlife and humans. Herring gulls (*Larus argentatus*) nesting near integrated steel mills on the Great Lakes were shown to have elevated heritable mutation rates, but the importance of airborne and aquatic routes of contaminant exposure could not be determined.

I showed that sentinel mice exposed to urban-industrial air pollution *in situ* near two integrated steel mills and a major highway had germline mutation rates at expanded-simple-tandem-repeat (ESTR) DNA loci that were 1.5- to 2.0-times as high as mice exposed at a cleaner rural site. In addition, high-efficiency-particulate-air filtration (HEPA) reduced mutation induction at the urban-industrial site, implicating a causal role for airborne particulate matter. These experiments identify an important role for air pollution exposure in the induction of heritable DNA mutations.

I developed and tested a novel diet medium for feeding high percentages of fish to mice for toxicology experiments. This diet was then used in a study of germline mutations in mice fed fish collected from a polluted industrial site, compared to those fed fish from Atlantic Canada, or control chow. There was a non-significant trend for mice that consumed fish from the polluted site to have higher germline ESTR mutation rates than those in the other groups.

For my experiments I used out-bred Swiss-Webster mice that had not been characterized previously in studies of germline ESTR mutations. I exposed male mice to ionizing radiation to determine whether germline mutation rates increased in a dose-

dependent manner following exposure to a known mutagen. Mutation rates did not increase linearly with radiation dose, showing evidence of saturation, but the estimate of doubling-dose was similar to that previously published for some inbred mouse lines.

PREFACE

This thesis consists of seven chapters. The first chapter is a general introduction, and the seventh is a synthesis and suggestions for future research. Chapters 2 through 6 have been written as manuscripts for publication in peer-reviewed scientific journals. At the time that this thesis was submitted, Chapters 2 and 3 were previously published (reprinted with permission), and Chapter 6 has been conditionally accepted for publication.

Chapters 4 and 5 are in preparation for submission to the journals *Toxicological Sciences*, and *Ecotoxicology*, respectively. Information about the title, authors, and individual contributions to each of the chapters is detailed below:

Chapter 2: "Air pollution induces heritable DNA mutations."

Authors: C.M. Somers, C.L. Yauk, P.A. White, C.L.J. Parfett, and J.S. Quinn
Reference: Proceedings of the National Academy of Sciences, USA, 2002, 99, 15904 – 15908.

Contribution: mouse breeding and all laboratory work were performed by the candidate under the guidance of Drs. J.S. Quinn and B.N. White. The statistical analyses and writing of the manuscript were conducted by the candidate with guidance and suggestions from the co-authors.

Chapter 3: "Reduction of particulate air pollution lowers the risk of heritable mutations in mice."

Authors: C.M. Somers, B.E. McCarry, F. Malek, and J.S. Quinn.

Reference: Science, 2004, 304, 1008-1010

Contribution: field exposures of lab animals were coordinated by the candidate. High-efficiency-particulate-air filtration chambers were designed by the candidate and constructed by R. Gillies. Mouse breeding, tissue sampling, DNA extraction, DNA fingerprinting, and all additional lab work were performed by the candidate with the assistance of a research technician. Collection of particulate air samples, and chemical extraction of organic material were performed by the candidate. Gas chromatography and mass spectrometry for quantification of individual chemicals were conducted by F. Malek under the supervision of B.E. McCarry. Statistical analyses were conducted by the candidate with direction from an anonymous reviewer, Dr. Y.E. Dubrova, Dr. S. Dudley, and J.S. Quinn. The manuscript and

all accompanying material were written by the candidate with suggestions from co-authors. The research was conducted under the supervision of J.S. Quinn.

Chapter 4: "An approach to feeding high percentage fish diets to mice for human and wildlife toxicology studies."

Authors: C.M. Somers, E. V. Valdes, and J.S. Quinn

Contribution: fish species selection and all animal trials were conducted by the candidate. The diet formulation was created by E.V. Valdes. The manuscript was written by the candidate with suggestions from co-authors. Statistical analyses were conducted by the candidate with guidance from Dr. S. Dudley. The research was conducted under the supervision of J.S. Quinn.

Chapter 5: "A diet of small-bodied fish and germline mutations at repetitive DNA loci in mice."

Authors: C.M. Somers, E.V. Valdes, V.A. Kjoss, A.M. Vallaincourt, and J.S. Quinn.

Contribution: collection of wild fish, preparation of all diet media, and all lab animal work was conducted by the candidate with the assistance of V.A. Kjoss. The fish-diet formulation was created by E.V. Valdes. All lab work was conducted by the candidate with the aid of volunteers and summer students. A.M. Vallaincourt performed contaminant analyses on fish specimens. The statistical analyses and manuscript writing were performed by the candidate with suggestions from co-authors. The research was conducted under the supervision of J.S. Quinn.

Chapter 6: "Gamma-radiation induced heritable mutations at repetitive DNA loci in out-bred mice."

Authors: C.M. Somers, R. Sharma, J.S. Quinn, and D.R. Boreham.

Reference: conditionally accepted for publication in *Mutation Research*.

Contribution: mouse irradiation and breeding were performed by the candidate under the guidance of D.R. Boreham. All lab work was conducted by the candidate, with the exception of a subsection done by R. Sharma as part of a 4th-year thesis project. Statistical analyses were performed by the candidate. The radiation doubling dose was estimated by Dr. Y.E. Dubrova. The manuscript was written by the candidate with suggestions from co-authors.

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CHAPTER 1
GENERAL INTRODUCTION

1.1 DNA-Damaging Agents in the Environment

Human activities have resulted in the release of large quantities of a diverse array of environmental contaminants (Shugart and Theodorakis 1998, Tornqvist and Ehrenberg 2001). Many chemical and physical agents that comprise environmental pollution have the capacity to induce genetic damage, and for some time there has been concern regarding evidence that contaminant exposure might be having a negative impact on the health of humans and wildlife (Nebert and Caravan III 1997, Sever 1997, Pollack 2003). In particular, both the scientific community and the general public have sought answers regarding whether or not environmental exposure to chemical or radioactive contaminants can cause cancer (Thilly 2003), or induce germline mutations that affect future generations (Kramer 1998). Unfortunately, early techniques in toxicology were often not sensitive enough to be informative in evaluating the relationship between genetic damage and environmental exposure, especially for complex mixtures (Logan and Wilson 1995). Relatively recent advances in molecular genetic techniques have resulted in the development of a suite of biological markers that offer greater sensitivity for investigating the genetic effects of contaminant exposure in both wildlife and human systems (DeCaprio 1997, Bickham et al. 2000).

1.2 Evidence for Induced Genetic Damage in Wildlife: Somatic Cells

Studies of genotoxicity in wildlife generally take the approach of comparing exposed populations living in contaminated areas to relatively unexposed populations living at more pristine locations. Biomarkers applied for this purpose have ranged from early warning signs of genetic damage, such as pre-mutational lesions, to screening for

chromosome abnormalities, DNA mutations, or tumours. In aquatic systems, this comparative approach has revealed elevated levels of DNA strand breaks in the somatic cells of fish living in areas contaminated with polychlorinated biphenyls (PCB) and polycyclic aromatic hydrocarbons (PAH; Pandrangi et al. 1995), and structural DNA damage in fish exposed to a mixture of heavy metals and radioactive cesium (Sugg et al. 1995). Subsequent studies found elevated levels of DNA strand breaks in a range of taxa, including molluscs, echinoderms, and amphibians exposed to mixtures of common contaminants in a variety of aquatic habitats (reviewed in Mitchelmore and Chipman 1998). Exposure to chemical contaminant mixtures has also been linked to lip tumour formation in benthic fish species in the Great Lakes and St. Lawrence River (Baumann et al. 1996, Mikaelian et al. 2000), liver tumours in marine fish of the Pacific Northwest (Myers et al. 1994), and all cancer types in St. Lawrence beluga whales (Martineau et al. 2002).

Similar comparisons have also been conducted among populations of terrestrial animals. White-footed mice (*Peromyscus leucopus*) living near a hazardous chemical clean-up training area in North America demonstrated higher frequencies of chromosome abnormalities in several tissues using DNA content heterogeneity measures (McBee and Bickham 1988, Tull-Singleton et al. 1994). Similar studies in Europe demonstrated elevated micronucleus and abnormal sperm cell frequencies in wild mice (*Mus domesticus*) exposed to air pollution near heavy traffic (Ieradi et al. 1996), and elevated micronucleus frequencies in four different species of wild rodents living near petrochemical factories (Degrassi et al. 1999). These studies have been contrasted by others that do not show convincing evidence for genetic damage to somatic cells. For

example, pigeons in urban environments showed increased levels of oxidative DNA damage in somatic cells, but did not show elevated levels of DNA adducts despite high PAH exposure from air pollution (Schilderman et al. 1997). Similarly, the embryos of black-headed gulls (*Larus ridibundus*) nesting in an area of Lithuania with high levels of air and water pollution did not show elevated micronucleus frequencies (Stoncius and Lazutka 2003), and black-crowned night-herons (*Nycticorax nycticorax*) living near petrochemical industries in North America did not show increased mutation rates in mitochondrial DNA, despite high PAH exposure (Dahl et al. 2001). In perhaps the most surprising example of a negative finding, bank voles (*Clethrionomys glareolus*) living in the most radioactive habitats in the world near the Chernobyl reactor showed only a marginal (non-significant) trend towards higher mitochondrial point mutation rates (Baker et al. 2001).

1.3 Evidence for Induced Genetic Damage in Wildlife: Heritable Mutations

Wildlife studies of heritable genetic effects are much more difficult to conduct because they require application of genetic biomarkers to at least two generations of animals. In addition, trans-generational effects are harder to detect because the sampling unit becomes pedigrees or individual offspring, as opposed to the potentially unlimited number of somatic cells available for general genotoxicity testing. Consequently, field studies of environmentally induced heritable mutations are extremely rare, and similar to studies using somatic cell biomarkers, they show mixed evidence for significant induction caused by different types of contaminant exposure. For example, Ellegren et al. (1997) demonstrated using pedigree DNA profiling at microsatellite loci that barn

swallows (*Hirundo rustica*) nesting in the highly radioactive area around Chernobyl had germline mutation rates that were 2- to 10-fold higher than those of unexposed reference populations. In addition, swallows in the radioactive area showed higher rates of heritable partial albinism. Similarly, wheat planted in contaminated fields near the Chernobyl reactor also had elevated germline microsatellite mutation rates compared to those of plants in unexposed reference fields (Kovalchuk et al. 2000). In contrast, tree swallows (*Hirundo rustica*) with extremely high dietary exposure to PCBs did not show elevated germline minisatellite mutation rates compared to those at cleaner reference sites (Stapleton et al. 2001), and pink salmon (*Oncorhynchus gorbuscha*) exposed to high levels of crude oil did not have elevated heritable mutation rates in the KRAS oncogene (Cronin et al. 2002).

The only direct evidence that mixtures of chemical contaminants can induce heritable mutations in wildlife comes from two studies of herring gulls (*Larus argentatus*) in the Great Lakes region of North America. In the first, pedigree DNA profiling at minisatellite DNA loci revealed significantly elevated mutation rates in a gull colony located in a polluted industrial area near two integrated steel mills (Yauk and Quinn 1996). An expansion of this study showed that mutation rates were consistently elevated in gull colonies near steel mills, and established a clear proximity relationship, with the highest mutation rates documented in colonies located closest to steel mills (Yauk et al. 2000). This research was the first to potentially link ambient levels of chemical pollution exposure with heritable mutations, but the type of pollution and route of exposure could not be determined. It was also not clear what potential effects differences among colonies, such as age structure, genetic background, and disease and

nutritional status, might have on comparisons of mutation rates (but see Yauk and Quinn 1999).

Overall, the evidence from wildlife studies clearly indicates that exposure to environmental radiation or chemical mixtures can pose a significant risk of induced somatic cell DNA damage, cancer, and heritable mutations. What remains unclear, however, is what levels, duration, and routes of exposure for different contaminant mixtures result in significant induction of genetic effects. This is especially true for heritable mutations, which have not been well studied in natural populations.

1.4 Evidence for Induced Genetic Damage in Humans: Somatic Cells

Investigations of genetic effects in humans have taken the same general approach as in wildlife studies where comparisons are made among relatively exposed and unexposed groups, and similar biomarkers have been applied. Although humans are routinely exposed to potentially genotoxic substances through a number of different routes (Neri et al. 2003), known mutagens and carcinogens are ubiquitous in urban and industrial air pollution, making airborne exposures a primary area of interest. Some epidemiology studies have identified significant associations between urban air pollution exposure and lung cancer (Arden Pope III et al. 2002), and industrial air pollution exposure and hematopoietic system cancers (e.g., Bhopal et al. 1998, Parodi et al. 2003). Other similar studies have not been able to detect any effect of air pollution on cancer rates (e.g., Harrison et al. 1999, Wilkinson et al. 1999), and the exposure conditions that result in health risks remain uncertain. Considerable controversy remains regarding the importance of ambient air pollution exposure for cancer risk (Krewski et al. 2003).

Statistical studies of disease incidence generally use indirect measures of pollution exposure, and do not permit mechanistic inference. It is therefore not clear whether the increased cancer rates discovered in the above studies resulted from a process beginning with DNA damage induced by genotoxic air pollution exposure.

More sensitive biomarkers have been applied to directly measure induced genetic damage in the tissues of humans exposed to a range of ambient air pollution conditions. Chromosome abnormalities and RAS oncogene expression were significantly elevated in the white blood cells of residents in areas of Poland with extremely high particulate and PAH air pollution levels (Perera et al. 1992). Similarly, visitors to highly polluted Mexico City showed elevated rates of DNA single strand breaks in nasal epithelial cells (Calderon-Garciduenas et al. 1996). In areas with lower but still problematic air pollution levels, bulky DNA adducts (Lewtas et al. 1997, Palli et al. 2001), chromosome abnormalities (Burgaz et al. 2002), and DNA base damage (Sorensen et al. 2003) were shown to be elevated in the white blood cells of humans exposed to particulate air pollution. Even using these more sensitive biomarkers, however, there has not been consistent correlation between different indicators of genetic damage and outdoor air pollution exposure measurements, nor have studies conducted in different geographic regions consistently shown that similar levels of air pollution exposure elevated genetic risks (Sram et al. 1999, Georgiadis et al. 2001, Kyrtopoulos et al. 2001).

1.5 Evidence for Induced Genetic Damage in Humans: Heritable Mutations

Studies of transmissible heritable mutations in humans have been limited to populations exposed to high levels of environmental radiation. Thorough development

and characterization of a suite of sensitive minisatellite DNA markers in humans have provided the best genetic tool for heritable mutation studies in any organism to date. Even with this efficient genetic biomarker, however, there has been little agreement among published studies regarding the effect of exposure on mutation rates. For example, Kodaira et al. (1995) did not find significantly elevated minisatellite DNA mutation rates in the offspring of Japanese parents exposed to high acute radiation doses from atomic weapons during the second world war. In contrast, a similar pedigree DNA profiling approach revealed significantly elevated germline minisatellite mutation rates in families exposed to low levels of chronic irradiation near the Chernobyl reactor, compared to reference populations in the United Kingdom or the Ukraine (Dubrova et al. 1996, 1997, 2002a). A similar effect was also found in residents of Kazakhstan exposed to chronic irradiation near nuclear weapons testing areas (Dubrova et al. 2002b). Studies of Chernobyl clean-up workers exposed during containment of the reactor add further confusion to this area of research. Weinberg et al. (1997) showed that the offspring of clean-up workers had significantly elevated rates of germline mutations at randomly amplified polymorphic (RAPD) DNA markers. This finding has been contradicted, however, by two independent studies that have not shown increased germline minisatellite or microsatellite mutation rates in similar cohorts of clean-up workers (Livshits et al. 2001, Slebos et al. 2004). There has been little consistency among all of the above studies in terms of the timing and level of radiation exposure, making it difficult to draw any conclusions regarding the risk of heritable mutation induction from environmental radiation.

Much less information is available regarding the relationship between heritable mutations in humans and exposure to chemical contaminants. No studies published to date on chemical exposure have taken the direct approach of genetic profiling in pedigrees as described above for radiation. Ambient air pollution is again a primary area of interest, given the number of exposed humans worldwide, and the near universal presence of chemical mutagens and carcinogens. A small number of studies have shown that chemical contaminant mixtures in ambient air pollution can cause genetic damage in germ cells, which suggests the possibility for elevated rates of transmissible mutations. For example, Perreault et al. (2000) showed a marginal (but not significant) increase in the frequency of sperm with altered chromatin structure in young men exposed to seasonal air pollution in the Czech Republic. Interestingly, despite the non-significant elevation in the more subtle biomarker, changes in sperm aneuploidy were significantly associated with air pollution exposure. This study was later expanded and showed clear evidence for seasonal effects of air pollution on DNA structural integrity in sperm (Selevan et al. 2000). There is currently no information available on which air pollutants caused these effects and at what levels. It is also unclear what the impact of these changes in sperm DNA might be on fertility, or the possibility of passing mutations to subsequent generations.

Similar to the situation with wildlife, human studies provide evidence that genetic damage can be induced in both somatic and germ cells by exposure to environmental radiation or chemical mixtures. It is not clear, however, what factors influenced the outcomes of studies that showed conflicting results. In the case of heritable mutations, there is clearly a lack of sufficient information available to evaluate specific risks posed

by exposure to ambient chemical mixtures in air pollution. There is also a puzzling lack of consistency among studies of radiation exposure. Establishing the types and levels of exposure important for induced genetic damage, particularly in germ cells, remains of paramount importance.

1.6 The Problem with Wildlife and Human Studies

Inconsistent findings in wildlife and human biomarker studies are not conducive to synthesis of the information in a way that could result in better environmental protection. This shortfall is due in a large part to fundamental difficulties inherent in the approach of comparing environmental exposure and genetic damage among existing natural populations. Below I briefly summarise three specific problems with wildlife and human studies, illustrated with examples.

(1) Underlying differences between exposed and reference populations (e.g., genetics, behaviour, lifestyle) can confound comparisons.

Differences between natural populations of organisms may exist that change their susceptibility to genetic effects induced by contaminant exposure, or that alter the frequency of biomarker events independent of the environmental exposure of interest. For example, in the groundbreaking study by Dubrova et al. (1996) that showed elevated germline minisatellite mutation rates in residents near Chernobyl, the unexposed group used for comparison was from the United Kingdom. Potential differences between these groups in terms of presence or absence of highly unstable minisatellite alleles (they were from different ethnic backgrounds), as well as lifestyle differences such as smoking and nutritional status, made attributing the observed elevation in germline mutation frequency

solely to radiation exposure somewhat problematic. This flaw in the study design resulted in the interpretations being hotly contested by other scientists (e.g., Neel 1999).

(2) Free-ranging subjects can move in and out of study areas, or in other ways modify their levels of contaminant exposure.

Movement between study areas can result in reduced asymmetry of exposure between 'polluted' and reference groups, obscuring the effects of contaminant exposure. For example, Theodorakis et al. (2001) were not able to show an overall elevation in the levels of micronucleus formation in tissues of kangaroo rats (*Dipodomys merriami*) exposed to radioactivity at nuclear weapons testing sites, compared to those unexposed at more pristine locations. Animals with mitochondrial DNA haplotypes unique to the contaminated sites, however, did show significantly elevated rates of somatic cell DNA damage compared to those with haplotypes common in surrounding uncontaminated areas. This suggested that movement of unexposed animals into the contaminated areas from outside was obscuring detection of genetic damage resulting from radiation exposure. This situation can be further confounding in human studies where subjects move frequently, or behave in unexpected ways. For example, Greek university students exposed to negligible air pollution in a small rural town had higher levels of PAH-DNA adducts in white blood cells than students living in a highly polluted area of Athens. Follow-up investigation revealed that behavioural differences resulted in the rural students being exposed to higher levels of indoor environmental tobacco smoke, effectively obscuring detection of any effect of outdoor air pollution (Georgiadis et al. 2001).

(3) Exposure to genotoxic contaminants can be through multiple routes, but their relative importance generally cannot be determined.

Most wildlife and human studies have used proximity to a potential source of pollution as a surrogate for direct measurement of exposure. Subjects in areas of interest are therefore likely to be exposed to contaminants through air, diet, water, and direct contact, but it is generally not possible to attribute any effects observed to a specific exposure route. For example, herring gulls nesting near steel mills on the Great Lakes were shown to have elevated germline minisatellite mutation rates (Yauk and Quinn 1996, Yauk et al. 2000). Aquatic habitats near industrial complexes containing steel mills are highly polluted with a range of contaminants, suggesting that gulls could be exposed to genotoxic chemicals through a contaminated fish diet. Industry and traffic in these same areas also release large quantities of airborne mutagens, suggesting that gulls could also be exposed to contaminants by breathing polluted air. In field studies of natural populations, however, it is impossible to specifically evaluate the relative importance of these two potential routes of mutagen exposure in inducing germline mutations.

1.7 The Solution? Sentinel Laboratory Animals in Toxicology Studies

A very simple and powerful experimental approach for environmental toxicology studies is to expose sentinel lab animals to ambient environmental conditions *in situ*. This approach allows for control over the three major problems identified above in comparative studies of free-ranging populations. Specifically, (1) laboratory animals from the same genetic stock can be exposed at multiple field sites, eliminating underlying genetic and behavioural differences between exposed and reference groups, (2) animals

can be confined at particular locations, eliminating the possibility of movement between polluted and unpolluted areas, and (3) potential routes of exposure to contaminants can be isolated and tested individually. This approach has been used widely by aquatic toxicologists, who place caged fish at specific locations in rivers to address questions regarding the toxicological effects of particular effluents (e.g., Harries et al. 1996, 1997, Tyler and Routledge 1998, Hewitt et al. 2000, Choi and Meier 2000). There have been no studies of heritable mutations in sentinel aquatic organisms, and relatively few attempts have been made to apply this approach to monitor any endpoint in terrestrial systems.

Given the number of humans potentially affected by air pollution, it is somewhat surprising that only a few sentinel animal studies have been conducted to evaluate the response of animals to ambient air exposure. Reichrtova et al. (1995) penned rabbits at a location adjacent to a mercury manufacturing plant in Slovakia for 6 months, and compared them to an identical group housed outside the polluted area. During exposure the researchers controlled sources of food and water for the rabbits so that air quality was the only factor that differed between sites. Using this approach, they were able to show definitively that air pollution near the plant increased the concentration of mercury in a variety of rabbit tissues, and caused significant pathological changes in the respiratory tract. In a similar program, researchers in Sao Paulo, Brazil, initiated lung tumours in rats in the laboratory using urethane, and then exposed the animals to ambient air pollution in either the heavily trafficked downtown portion of the city or in a rural location 65 km away. Following six months of exposure, lung tumours in the rats exposed downtown showed significant changes in tumour phenotype, and advanced progression towards malignancy compared to their rural counterparts (Saldiva et al. 1995, Reymao et al. 1997,

Curry et al. 2000). Follow-up work to this showed that untreated mice housed at the same urban and rural locations had significantly elevated micronucleus frequencies in peripheral erythrocytes, showing that urban air pollution can induce physical DNA damage in somatic cells (Soares et al. 2003).

An extension of the idea of exposing sentinel animals *in situ*, as described above, is to bring outdoor conditions into the laboratory. This is an extremely difficult task for air pollution studies, which can never recreate the complexity of outdoor chemical mixtures. Where it is potentially applicable, however, is in the study of diet items. For example, rather than attempting to recreate the complex mixtures of accumulated organic contaminants in sport fish tissues, toxicologists have conducted studies in which wild fish were fed directly to laboratory animals (e.g., overview of the major Health Canada study described in Arnold et al. 1998). Experimental endpoints covered in these feeding studies have included a range of physiological measurements relevant to development and reproduction (e.g., Cleland et al. 1987, Restum et al. 1998), but no studies have applied biomarkers of genotoxicity to examine the potential for induced genetic damage.

Despite the apparent advantages of the sentinel lab animal approach, it has only recently been used to study somatic mutations induced by ambient exposure to environmental radiation (Wickcliffe et al. 2003), and no studies to date have attempted to use sentinel animals to study heritable mutations induced by environmental contamination of any kind. Given our current lack of understanding of the relationship between ambient environmental exposures and mutation frequency in germ cells, sentinel lab animal experiments offer a unique opportunity to advance knowledge in this area of environmental toxicology.

1.8 Thesis Objectives

Studies of heritable mutations induced by exposure to mixtures of chemical contaminants under ambient environmental conditions remain rare. To date the only evidence that transmissible germline mutations can be induced by chemical contaminant exposure comes from studies of herring gulls nesting in polluted industrial areas near steel mills on the Great Lakes (Yauk and Quinn 1996, Yauk et al. 2000). These studies were not able to determine the relative importance of airborne or aquatic contaminant exposure, thus making it impossible to predict whether other organisms in similar habitats, including humans, could also be affected.

During approximately the same time period that herring gulls were being studied in North America, researchers in the United Kingdom were developing and applying a new class of DNA markers called expanded-simple-tandem-repeats (ESTR) for laboratory studies of induced germline mutations in mice (Kelly et al. 1989, Gibbs et al. 1993, Bois et al. 1998). These repetitive DNA loci are the most efficient genetic marker system ever developed for studying germline mutations in rodents, allowing detection of statistically significant effects using fewer offspring and lower treatment doses than any previous technique (Dubrova et al. 1993, Hedenskog et al. 1997, Dubrova et al. 1998, Vilarino-Guell et al. 2003).

The primary objective of my thesis was to determine the relative importance of airborne and aquatic routes of chemical contaminant exposure for the induction of heritable mutations at a polluted industrial site near integrated steel mills. Specifically, I combined the experimental approach of exposing sentinel lab animals to ambient

environmental conditions, with the sensitive genetic technique of pedigree DNA profiling at ESTR loci, to test two hypotheses: (1) germline ESTR mutations are induced in mice by exposure to urban-industrial air pollution, and (2) germline ESTR mutations are induced in mice by a diet of contaminated fish. For my environmental exposure experiments with sentinel mice I focused on Hamilton Harbour, a polluted industrial site on western Lake Ontario that consistently showed elevated minisatellite mutation rates in previous studies of herring gulls.

Testing the second hypothesis described above required feeding mice a diet very high in fish content. The fish species to be used were novel for rodent feeding studies, and a second objective of my thesis became the development of a diet for mice that allowed incorporation of high percentages of fish, while remaining palatable and nutritionally balanced.

In all of my experiments I used out-bred Swiss-Webster mice that had not been previously characterized at ESTR loci. The third objective of my thesis was to determine whether germline ESTR mutations could be induced in this mouse strain in a dose-dependent manner following exposure to a known mutagen. Specifically, I exposed mice to doses of ionizing radiation that had been used previously in studies of inbred mouse lines, to test the hypothesis that mutation rates in out-bred mice increase in a linear fashion with radiation dose.

The overall goal of this work was to determine the utility of the sentinel mouse / ESTR DNA profiling approach for studies of heritable mutations induced by environmental contaminant exposure. In addition, I sought to provide further insight into

issues remaining from herring gulls studies, including providing more information on the risk of germline mutations for other organisms living in polluted industrial areas.

1.9 References

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CHAPTER 2

Air Pollution Induces Heritable DNA Mutations

Classification: Biological Sciences, Applied Biological Sciences

*Christopher M. Somers, [†]Carole L. Yauk, [†]Paul A. White, [†]Craig L. J. Parfett, and
*James S. Quinn

*McMaster University, Department of Biology, 1280 Main St. West, Hamilton, Ontario,
Canada, L8S 4K1

[†]Mutagenesis Section, Environmental and Occupational Toxicology Division, Health
Canada, Environmental Health Centre, 0803A, Tunney's Pasture, Ottawa, Ontario,
Canada, K1A 0L2

Corresponding Author:

Dr. James S. Quinn
McMaster University, Department of Biology, 1280 Main St. West, Hamilton,
Ontario, Canada, L8S 4K1
Phone (905) 525 – 9140 x 23194
Fax (905) 522 – 6066
quinn@mcmaster.ca

Abbreviations: ESTR = expanded simple tandem repeat DNA

ABSTRACT

Hundreds of thousands of people worldwide live or work in close proximity to steel mills. Integrated steel production generates chemical pollution containing compounds that can induce genetic damage (1, 2). Previous investigations of herring gulls in the Great Lakes demonstrated elevated DNA mutation rates near steel mills (3, 4), but could not determine the importance of airborne or aquatic routes of contaminant exposure, nor eliminate possible confounding factors such as nutritional status and disease burden. To address these issues experimentally, we exposed laboratory mice *in situ* to ambient air in a polluted industrial area near steel mills. Heritable mutation frequency at tandem-repeat DNA loci in mice exposed 1 km downwind from two integrated steel mills was 1.5- to 2.0-fold elevated compared to those at a reference site 30 km away. This statistically significant elevation was due primarily to an increase in mutations inherited through the paternal germline. This is the first demonstration of heritable mutations induced in any organism following a controlled exposure to ambient air. Our results indicate that human and wildlife populations in proximity to integrated steel mills may be at risk of developing germline mutations more frequently due to the inhalation of airborne chemical mutagens.

INTRODUCTION

Integrated steel mills produce chemical mutagens that contaminate atmospheric and aquatic environments (1, 2), and may pose a genetic hazard to humans and wildlife. Herring gulls (*Larus argentatus*) nesting near steel mills on the Great Lakes were shown to have higher germline mutation rates at minisatellite DNA loci than those at rural sites (3), and mutation frequency increased with colony proximity to integrated steel mills (4). It was postulated that inhaled airborne contaminants emitted from steel mills, such as polycyclic aromatic compounds, were largely responsible for mutation induction; however, contaminants in the aquatic food web, and differences in disease and nutritional status among gull colonies could not be eliminated as contributing factors. Therefore, the role of air pollution in producing germline mutations, and the risk to humans living near steel mills could not be determined.

Rodent expanded-simple-tandem-repeat (ESTR) DNA consists of 4 to 6 base-pair repeat units in long tandem arrays that are unstable in the germline, and tend to mutate by insertion or deletion of a number of repeat units (5, 6, 7). Laboratory studies have demonstrated that murine ESTR loci are susceptible to germline mutations induced by chemical (8) or radioactive (9, 10) mutagens, and therefore may be useful tools for environmental contamination studies. The use of sentinel laboratory animals exposed *in situ* is a powerful experimental approach for assessing air pollution hazards because it combines the controlled elements of laboratory studies with direct exposure to ambient pollution levels (11, 12). Here we compare germline ESTR mutation rates in laboratory mice exposed to ambient air at an industrial site near integrated steel mills to those

exposed at a rural reference location, with the objective of testing inhalation of industrial air pollution as a route of chemical mutagen exposure.

MATERIALS AND METHODS

Environmental Exposure - We housed groups of laboratory mice at two field sites: 1 km downwind from two integrated steel mills in Hamilton Harbour (43°15'N, 79°51'W), a polluted industrial area on western Lake Ontario (steel), and simultaneously at a rural reference location 30 km away (rural). The steel site was chosen based on elevated germline mutation rates in local herring gull colonies (3, 4) and high levels of chemical mutagens, such as polycyclic aromatic hydrocarbons (PAH), present in the air (1).

At each site 20 male and 20 female outbred Swiss-Webster mice, 6- to 8-weeks old (Charles River Laboratories, St. Constance, Quebec, Canada), were housed 5 same-sex individuals per cage inside of identical 2.4 x 2.4 x 1.8 m vinyl utility sheds. Sections of shed walls (1.2 x 1.6 m) were replaced with hardware cloth (1.5 x 1.5 cm mesh), which allowed flow-through of ambient air. We erected both sheds on the same compass orientation in partial shade to keep sun exposure time and direction constant at each site. We placed mouse cages on a single shelving unit inside of each shed such that they were not exposed to direct wind or sunlight. Exposures lasted 10 weeks from 10-September to 21-November-1999, during which mice at both sites were given commercial mouse chow and bottled water from the same source.

Minimum temperature in each shed was partially controlled using electric heaters. We monitored temperature range on 53 of the 70 days of exposure using a max. / min. thermometer at the rural site, and data from a nearby weather station at the steel site.

Average maximum and minimum temperatures were 18.6 ± 4.7 , 6.9 ± 3.8 °C and 16.8 ± 5.1 , 8.5 ± 4.4 °C for the rural and steel sites respectively. Temperature did not fall below negative 3 °C, or rise above 29 °C at either site.

Mouse Breeding - Following *in situ* exposure mice were returned to the animal care facility at McMaster University where they were given unique tail tattoos that identified individuals and exposure location. To ensure that fertilizations resulted from mature sperm that developed from 2n-spermatogonia beginning during *in situ* exposure, mice were held in same-sex groups for 6 weeks post-treatment (13, 14, 15). Breeding pairs were then assigned randomly within each group. We monitored females daily beginning 18 days post-pairing, and recorded date of delivery and litter size, as well as mass of pups at 4 intervals during the first 5 days of growth. Tail tissue was sampled from complete families when the pups were five days old. All animal procedures were approved by the McMaster University Animal Research Ethics Board following the guidelines of the Canadian Council on Animal Care.

Genetic Analysis - Genomic DNA was extracted from tail tissue of both parents and 3 to 6 pups from each mouse family using a standard phenol / chloroform procedure. DNA (6 µg) was digested to completion with Hae III, size-fractionated in 42-cm long, 0.8 % agarose gels for 42 to 50 hours (1.5 to 2.5 volts/cm), and transferred to nylon membrane by Southern blotting (Hybond-XL, Amersham-Pharmacia). DNA fingerprints were generated by sequential hybridization with ³²P-labelled synthetic ESTR probes Ms6-hm (5), Hm-2 (6), and MMS10 (7), and visualized by autoradiography. Blots were

completely stripped of probe DNA between hybridizations using 42 °C, 0.4 M NaOH, followed by boiling 0.1 % SDS. All samples were run with 30 ng of digested lambda phage DNA as an in-lane size standard (16).

Fingerprint bands in offspring that deviated by 1.0 mm or more relative to the parental progenitor, as determined using the in-lane size standard, were scored as mutations (17). All bands co-detected by both a single and multilocus ESTR probe were included in the single locus mutation rate only. Mutation events resulting in identical mutant bands shared among littermates (clustered mutations), or extra single locus bands (somatic mutations during embryogenesis), were not included in our analyses. Scoring was performed without knowledge of exposure location, and verified by an independent observer. Mutation rates were calculated as the number of mutant bands out of the total scored, and compared using a one-tailed Fisher's Exact Probability Test. Parentage of all pups was confirmed with band-sharing using multilocus probe MMS10.

RESULTS

Twenty breeding pairs were established from mice exposed at each field site. Seventeen pairs of steel mice and 19 pairs of rural mice successfully produced offspring (85% and 95% breeding success for steel and rural respectively). Litters born to steel parents had 1.7 fewer pups on average than rural (mean litter size 7.9 ± 2.6 and 9.6 ± 3.0 pups for steel and rural, respectively; Mann-Whitney, $n_1 = 19$, $n_2 = 17$, $U = 230.5$, $P = 0.07$). The mass of pups from each group did not differ significantly at any time over the first 5 days of growth (data not shown).

Single and multilocus ESTR probes detected a 1.5- and 2.0-fold elevation in germline mutation frequency in the steel group over the rural group, respectively (Table 1). Examples of mutant bands detected with ESTR single locus probe Hm-2 are shown in Figure 1. Multi-alleleism and high heterozygosity at ESTR single loci Ms6-hm and Hm-2 allowed us to determine the parental origin of mutant bands in all cases. Paternal mutations were 1.6-times more frequent in steel mice than rural, making up most of the overall elevation in the steel group (Table 2). Maternal mutation rates did not differ significantly between the two sites.

DISCUSSION

We placed laboratory mice in a high-risk area for induced germline mutations as identified during herring gull studies (3, 4), and controlled for sources of environmental mutagens other than airborne emissions. Identical husbandry conditions for mice in both treatment groups during the study eliminated differences in nutritional and developmental history of the animals (average mass of adults did not differ between sites). We therefore attribute the effect on inherited mutations in the offspring of sentinel mice directly to variation in air quality between the steel and rural field sites. This is the first demonstration of heritable mutation induction in any organism as a result of ambient air pollution exposure.

Few other studies have been able to establish a significant relationship between ambient pollution levels and germline mutation induction. Hypervariable repetitive DNA markers revealed elevated mutation rates in human families living near radioactive contamination sites in Belarus (18, 19), Kazakhstan (20), and the Ukraine (21), as well as

barn swallows nesting near Chernobyl (22). These sites, however, are unusual in that the source of mutagenic pollution was either a nuclear reactor accident (18, 19, 20) or bomb testing (22), which are uncommon events in most human populated areas. Elevated minisatellite mutation rates in herring gulls nesting near steel mills on the Great Lakes (3, 4), and our findings in this study are currently the only examples of heritable mutation induction from chemical pollution sources. In contrast to previous studies, urban and industrial air pollution has the potential to affect many people in most countries. Taken in concert with our observation of a marginal decrease in litter size in steel mice, which may be indicative of dominant lethal mutations (23), our findings suggest that there is an urgent need to investigate the genetic consequences associated with exposure to chemical pollution through the inhalation of urban and industrial air.

The highest germline mutation rate we observed in sentinel mice was in males exposed at the steel site. The timing of spermatogenesis in mice and the 6-week delay in breeding following *in situ* exposure indicate that premeiotic male germ cells (24, 10) are sensitive to airborne emissions near steel mills. This period during male gametogenesis is also sensitive to ESTR mutations induced by ionizing radiation (10, 25). The fact that the male germline mutation rate was significantly elevated in our study after only a ten-week exposure is reason for concern. Globally, hundreds of thousands of humans live or work in industrial areas near steel mills, and are incidentally exposed to airborne emissions. These populations may be at risk of increased heritable mutation frequency through exposed fathers. In addition, steelworkers in certain positions inside plants can be exposed to airborne emissions at much higher levels than outdoor ambient (26). Many

more men than women work in steel mills, raising the possibility of heritable mutation as a previously undocumented occupational hazard.

In contrast, the slight elevation in maternal mutation rate we detected at the steel site was not statistically significant. It would be premature based on this to conclude that the female germline is not at risk from air pollution exposure. Relatively little is known about the induction of heritable mutations in female germ cells; however, mutation induction in mature oocytes has been demonstrated using the specific locus test with both radioactive and chemical mutagens (reviewed in 23, 27). To our knowledge, direct ESTR mutation induction in the female germline using any sort of mutagen has never been investigated (see 29, 30 for examples of indirect maternal ESTR mutation induction). We suggest that future research address the issue of ESTR mutation induction in females. Of particular interest would be long-term, low dose exposure of adults, and embryonic exposures encompassing gametogenesis.

For management purposes it would be most pertinent to identify important chemical mutagens and restrict their release into the air. At the present time, however, we can only state that some portion of the air in Hamilton Harbour (steel site) caused elevated heritable mutation frequency. PAHs are produced in large quantities by integrated steel mills (31), tend to be associated with breathable particulate matter (32), and are among the most genotoxic components of urban air pollution (33). Total PAH concentration in Hamilton Harbour air (average for 1999 = 117.6 ng/m^3) was similar to that found to cause genotoxic effects in human white blood cells (32), and more than 50-times higher than that of the county containing the reference site (F. Dobroff, Ontario Ministry of the Environment, *pers. comm.*). In addition, extracts of particulate matter

containing PAHs in Hamilton air were shown to be mutagenic in a dose-dependent manner using the Ames test (1). We suggest that PAHs from incomplete coal combustion during steel production are a likely candidate group for causing elevated germline mutation rates in the steel mice exposed in our study.

Integrated steel mills in Hamilton are not the only local source of PAHs and other toxic airborne emissions. A nearby major commuter highway, diesel-powered industrial vehicles, and the surrounding population of over 640 000 humans also contribute to air pollution in our study area. It is important to note, however, that Yauk *et al.* (4) examined germline mutations in a herring gull colony in Toronto, a major city of 3 million people that has a high volume of traffic, but no integrated steel mills. They found the mutation rate in the Toronto colony to be intermediate between rural and steel sites, suggesting that steel mills contribute significantly to germline mutation induction.

The possible impacts on human health associated with increased mutation rates in repetitive DNA sequences are not known; however, ESTR mutations show a dose-response relationship to radiation exposure, and have a similar doubling dose to coding regions of DNA (10). It is therefore likely that a relationship exists between mutation frequency at ESTR loci and coding regions that affect phenotype. In addition, ESTR mutations are induced through unknown mechanisms that may alter DNA replication, recombination, or repair, and thus adversely affect the entire genome. Our results suggest that a thorough investigation of the genetic hazards associated with occupational and incidental exposure to contaminated air in urban and industrial areas is warranted.

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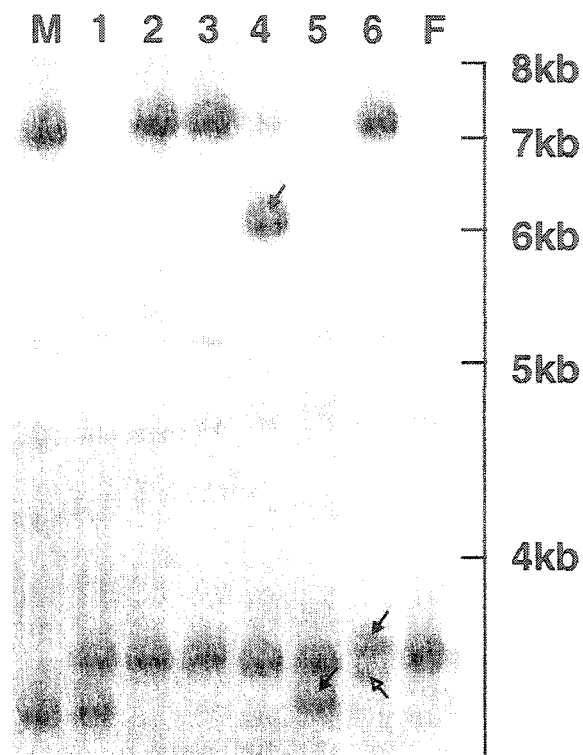


Fig 1. DNA profile of a mouse family exposed at the steel site at ESTR locus Hm-2. The parents are labelled with M (male) and F (female); pups are marked 1 through 6 (size range is indicated in kilobases). Three germline mutations are identified with solid arrows. The paternal alleles in pups 4 and 5 have undergone a large reduction and a small gain in size, respectively. The maternal allele in pup 6 has undergone a germline mutation event resulting in a small increase in size (solid arrow), as well as a somatic mutation during embryogenesis producing a less intense extra band (open arrow). Somatic mutation events were not included in our analyses

Table 1 Germline DNA mutation rates in sentinel mice exposed *in situ*.

Site	Probe	Pups scored	Bands scored	Mutant bands	Mutation rate per band ($\pm$ SE [†])	Fisher's Exact Test P-value [‡]
Rural	Ms6-hm	110	234	51	0.22 $\pm$ 0.03	
	Hm-2	96 [§]	150	23	0.16 $\pm$ 0.03	
	Total single locus:		384	74	0.19 $\pm$ 0.02	
	MMS10	110	1851	51	0.03 $\pm$ 0.00	
Steel	Ms6-hm	94	188	50	0.27 $\pm$ 0.03	0.06
	Hm-2	75 [§]	96	30	0.31 $\pm$ 0.05	0.01
	Total single locus:		284	80	0.28 $\pm$ 0.03	0.01
	MMS10	94	1523	93	0.06 $\pm$ 0.01	< 0.01

* The mutation rates presented do not include 7 and 12 instances of identical mutant bands shared among two or more littermates (clustered mutations) in the rural and steel groups, respectively. The frequency of this type of mutation event did not differ between groups (two-tailed Fisher's Exact Test, $P = 0.28$).

[†] SE = $[p(1 - p) / n]^{0.5}$, where p = observed mutation rate

[‡] Probability value for a one-tailed Fisher's Exact Test comparing Rural and Steel mutation rates

[§] Hm-2 fragments were too small to be detected in a number of parents, resulting in a reduction in the number of pups and bands screened for mutations at this locus

Table 2. Paternal and maternal germline ESTR mutation rates in sentinel mice exposed *in situ*. Data for single loci Ms6-hm and Hm-2 are presented combined. Footnotes are as in Table 1.

Site	Band Origin	Mutant Bands	Bands Scored	Mutation rate* per-band ($\pm$ SE †)	P-value ‡
Rural	Paternal	38	192	0.20 ± 0.03	
	Maternal	36	192	0.19 ± 0.03	
Steel	Paternal	46	142	0.32 ± 0.04	0.01
	Maternal	34	142	0.24 ± 0.04	0.07

CHAPTER 3

Reduction of Particulate Air Pollution Lowers Risk of Heritable Mutations in Mice

Christopher M. Somers*, Brian E. McCarry[⊥], Farideh Malek[†], James S. Quinn*[‡]

* McMaster University, Department of Biology, 1280 Main Street West, Hamilton, Ontario, Canada, L8S 4K1

⊥ McMaster University, Department of Chemistry, 1280 Main Street West, Hamilton, Ontario, Canada, L8S 4M1

† Lakeland College, School of Environmental Sciences, 5707-47 Avenue West, Vermilion, Alberta, Canada, T9X 1K5

‡ To whom correspondence should be addressed (e-mail: quinn@mcmaster.ca).

ABSTRACT

Urban and industrial air pollution can cause elevated heritable mutation rates in birds and rodents. The relative importance of airborne particulate matter versus gas-phase substances in causing these genetic effects under ambient conditions has not previously been determined. Here we show that high-efficiency-particulate-air (HEPA) filtration of ambient air significantly reduced heritable mutation rates at repetitive DNA loci in mice exposed outdoors in an urban-industrial area near two integrated steel mills and a major highway. Our findings implicate exposure to airborne particulate matter as a principal factor contributing to elevated mutation rates in sentinel mice, and add to accumulating evidence that air pollution may pose genetic risks to humans and wildlife.

Air pollution has the potential to affect millions of humans worldwide, and has been associated with an increased risk of lung cancer (e.g., 1) and genetic damage in other tissues (e.g., 2-5). To investigate whether air pollution induces heritable DNA mutations, we previously exposed sentinel laboratory mice *in situ* to ambient air for 10 weeks at two field sites: one was located in an urban-industrial area near two integrated steel mills and a major highway on Hamilton Harbour (Ontario, Canada), and the other was in a rural location 30 km away. Comparison of germline mutation rates at expanded-simple-tandem-repeat (ESTR) DNA loci in mouse pedigrees from each site revealed a 1.5 to 2.0-fold increase in mutation rate at the urban-industrial site, providing evidence that air pollution can cause genetic damage in germ cells, inducing trans-generational effects (6). We could not, however, identify causative agents or potential approaches for reducing mutation risk in urban and industrial areas.

To address these issues, in this study we housed two groups of sentinel lab mice concurrently for 10 weeks at the urban-industrial site described above (6). The first group was exposed to ambient air, whereas the second group was housed inside of a chamber that received HEPA-filtered air exclusively. HEPA-filtration removes at least 99.97% of particles 0.3 μm in diameter (7), and the system we used is rated by the manufacturer to remove up to 99.99% of particles down to 0.1 μm in diameter. In addition, HEPA filtration substantially reduces levels of even smaller ambient particles, down to 0.01 μm (8). Mice inside the HEPA-filtration chamber were therefore protected from exposure to all airborne particulate matter, with the exception of the smallest ultra-fine particles. We simultaneously housed mice under identical treatment conditions at a rural location 30 km away (rural site)

for comparison. Nine weeks after concluding the exposure, we bred the mice and compared germline mutation rates among groups using pedigree DNA profiling at ESTR loci (9 - 12).

Extensive polymorphism at ESTR loci Ms6-hm and Hm-2 allowed us to determine the parental origin of all mutant bands (detailed summaries of mutation data are available in Tables S1 and S2). The offspring of mice exposed to ambient air at the urban-industrial site inherited ESTR mutations of paternal origin 1.9- to 2.1-times more frequently than the offspring in any of the other three main treatment groups (Fig. 1A). Mice exposed to HEPA-filtered air at the urban-industrial site had paternal mutation rates that were 52% lower than those exposed to ambient air at the same location (Fig 1A). Exposure site and HEPA-filtration treatment, as well as the interaction between these two variables, explained a significant proportion of the variance in paternal mutation rates (Table 1). In contrast, maternal mutation rates were not significantly affected by either exposure site or HEPA-filtration (Table 1). When males exposed to ambient air at the urban-industrial site were mated to unexposed females, their offspring inherited ESTR mutations of paternal origin 2.8-times more frequently than those of rural males mated to unexposed females (Fig. 1B; Table S2). In this case the main effects of site and sex were significant, but there was not a significant interaction between the two variables (Table S3).

HEPA-filtration of ambient air therefore reduced ESTR mutation rates at the urban-industrial site, indicating that airborne agents removed by the HEPA filter were necessary for mutation induction. HEPA-filtration does not affect gas-phase substances, so the elevation in germline mutation rate in mice exposed at the urban-industrial site must have been caused by exposure to ambient air containing particulate matter larger than 0.01 to 0.1 μm . Furthermore, this particulate exposure affected ESTR mutation induction primarily

in the paternal germline (Fig 1A, B). The timing of spermatogenesis in mice and the 9-week delay in breeding following environmental exposure indicate that particulate air pollution affected pre-meiotic male germ cells (13). This developmental stage was also sensitive to environmental stresses in previous field (6) and laboratory (14 – 16) studies of ESTR mutations. The borderline (near significant) effect of exposure site observed for maternal mutation rates (Table 1) is similar to our previous findings (6), and suggests that further studies examining ESTR mutation processes in the maternal germline are necessary.

We measured levels of airborne particulate matter at both field sites on the same twenty five days during mouse exposures. Samples were grouped for analysis based on wind direction and the location of sentinel mice at the urban-industrial site relative to the industrial core area of the city of Hamilton. Samples collected on the same dates at the rural site were similarly grouped for comparison. Mean total suspended particulate (TSP; consists of respirable fraction as well as larger particles) was higher at the urban-industrial site than the rural site (2- to 10-fold; Wilcoxon Signed-Rank, $P = 0.036$), and was associated with the daily number of hours that mice were downwind of the industrial core area (Table 2; Kendall's $\tau = 0.733$, $P = 0.039$). To examine whether this relationship held true for a group of chemical mutagens commonly associated with airborne particulate, we quantified levels of 26 polycyclic aromatic hydrocarbons (PAH), including the seven carcinogens identified by the U.S. Environmental Protection Agency (Table S4). Total PAH concentrations were elevated at the urban-industrial site over the rural site (Wilcoxon Signed-Rank, $P = 0.036$), and were associated with the daily number of hours the sampler was downwind of the industrial core area (Fig. 2; Kendall's $\tau = 1.00$, $P = 0.005$). This relationship was much more pronounced for PAHs than for TSP, with PAH levels elevated

from 4- to 171-fold at the urban-industrial site, depending on wind direction (17). The weighted-average daily PAH exposure was 33-fold higher at the urban-industrial site than the rural site, at 13.4 ng/m³ and 0.4 ng/m³, respectively.

How exposure to airborne particulate matter induces genetic changes in the male germline is unknown. Automobile traffic and integrated steel production generate airborne mutagens, including PAHs (18) and heavy metals, and particles may deliver these chemicals and their metabolites to the bloodstream and ultimately the germ cells through the respiratory system. The relationship between PAH concentration and wind direction we quantified at the urban-industrial site indicates a source of emissions from the industrial area, and total PAH levels were relatively high (about 5- to 30-times higher than in Toronto, Canada's largest city). In addition, benzo[a]pyrene, a potent mutagen and carcinogen often used for assessing risk from PAH exposure, exceeded the recommended lifetime cancer risk guideline (19) of 0.1 ng/m³ by as much as 27-fold during 81% of mouse exposure days. If PAHs contribute to mutation induction, then the PAH-removal efficiency of our HEPA unit may be an important consideration. Based on chemical analysis of HEPA filters compared to TSP samples from the same location, HEPA-filtration blocked 7.1 to 8.0 ng/m³ (55% to 61%) of total PAHs in ambient air at the urban-industrial site. This is likely to be an underestimate (supporting online text), and HEPA-filtration probably removed 55% to 100% of particulate-bound PAHs, substantially reducing exposure for the sentinel mice. PAHs may contribute to germline mutation induction, but we cannot make specific conclusions regarding their importance at this time.

Human epidemiological studies have associated air pollution exposure with negative health consequences, including cardiovascular (20), respiratory (21), and

developmental impairments (22, 23), and lung cancer (1). Identification of the most dangerous air pollutants and their mode of action in producing specific health effects remains uncertain (24). Our study identifies airborne particulate matter as an important contributor to heritable mutation induction in mice; however, a direct link between ESTR mutations and health effects has not yet been established. In addition, although elevated germline mutation rates have been documented in both birds (25, 26) and mice (6) near industrial areas, it is not clear whether our results can be extrapolated to humans (but see 27). Data from mouse studies (15, 16) suggest that a relationship may exist between mutation rates at ESTR loci, and those in coding regions of the genome that affect phenotype. To reduce the potential risk of harmful heritable mutations for humans and wildlife, along with a suite of other health problems, we suggest that steps be taken to reduce levels of airborne particulate matter in urban environments.

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28. We thank K. and G. Gourlay for housing the rural mice on their farm; the Hamilton Port Authority for access to their property; V. Kjoss and S. Kassim for technical support; J. Seaman for flow rate data on the HEPA filtration units; F. Dobroff for weather data from Hamilton Harbour; Y. E. Dubrova, S. Dudley and an anonymous reviewer for statistical advice; and C. L. Yauk for comments on the manuscript. Supported by funds from the Toxic Substances Research Initiative and the Natural Sciences and Engineering Research Council of Canada.

Supporting Online Material, www.science.mag.org, Materials and Methods, Results, Tables S1 to S4, References and Notes.

Table 1. Summary of two-way ANOVA results for the effect of environmental exposure treatment on per-family paternal and maternal single locus ESTR mutation rates.

Source	<i>df</i>	Paternal		Maternal	
		<i>F</i> -value	<i>P</i> -value	<i>F</i> -value	<i>P</i> -value
Exposure site	1, 69	7.22	0.0090	3.68	0.0590
HEPA-filtration	1, 69	8.03	0.0060	0.07	0.7948
Interaction	1, 69	13.79	0.0004	1.60	0.2098

Table 2. Mean ($\pm$ SD) total suspended particulate (TSP) measured at the rural and urban-industrial sites. Samples collected at the urban-industrial site were grouped into six categories based on the daily number of hours sentinel mice at this location were downwind of the industrial core area. Samples collected on the same dates at the rural site were similarly grouped for comparison. The number of samples collected and the percentage of days during the 10-week mouse exposure with similar downwind times are indicated.

Daily time downwind of industrial core (hours)	Sample size	Rural Site Mean TSP ($\mu\text{g}/\text{m}^3$)	Urban- Industrial Site Mean TSP ($\mu\text{g}/\text{m}^3$)	% exposure days
0	5	16.2 ± 8.3	38.9 ± 10.5	13
1 to 3	4	23.5 ± 9.3	47.0 ± 17.1	16
4 to 9	3	32.5 ± 9.5	58.5 ± 29.2	14
10 to 18	4	41.9 ± 19.5	127.2 ± 76.6	20
19 to 23	3	10.8 ± 6.3	109.7 ± 35.4	13
24	6	31.7 ± 13.2	115.3 ± 25.3	24

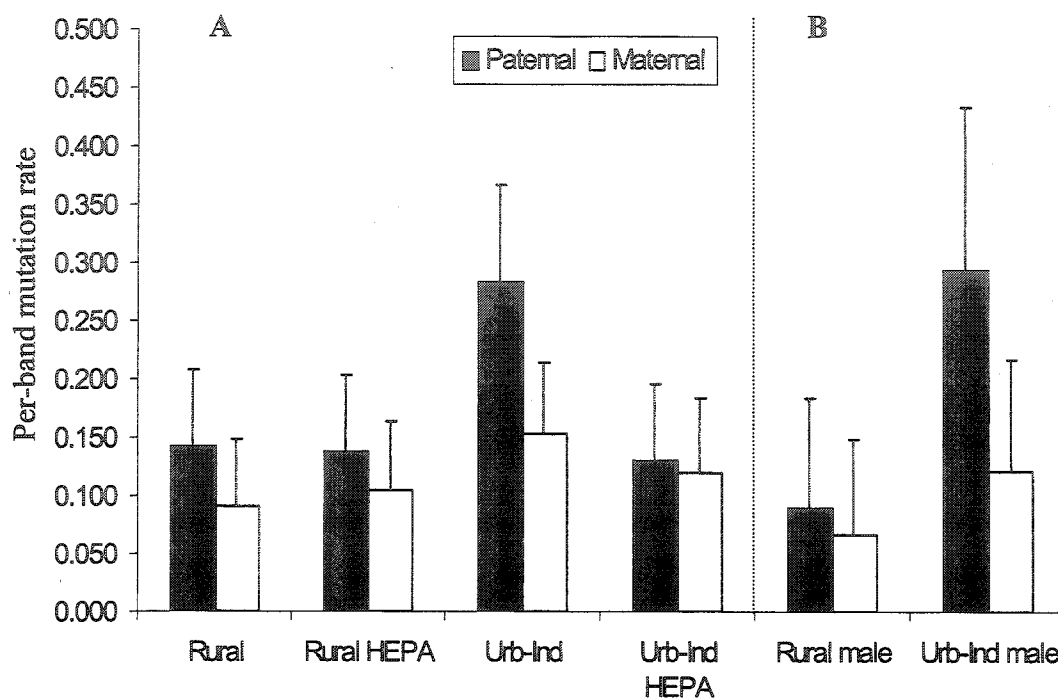


Fig. 1. (A) The paternal and maternal per-band mutation rates (+ 95% CI) measured in the offspring of sentinel mice exposed in situ to ambient or HEPA-filtered ambient air at the urban-industrial (Urb-Ind) and rural field sites. Mutation rates are for ESTR single loci Ms6-hm and Hm-2 pooled, and are based on analysis of 94 to 114 offspring (177 to 220 bands) in 17 to 20 pedigrees from each treatment group. (B) Mutation rates resulting from mating males exposed to ambient air at the rural and Urb-Ind sites to unexposed females. Mutation rates are for Ms6-hm and Hm-2 pooled from 8 pedigrees (45 to 48 offspring, 79 to 91 bands).

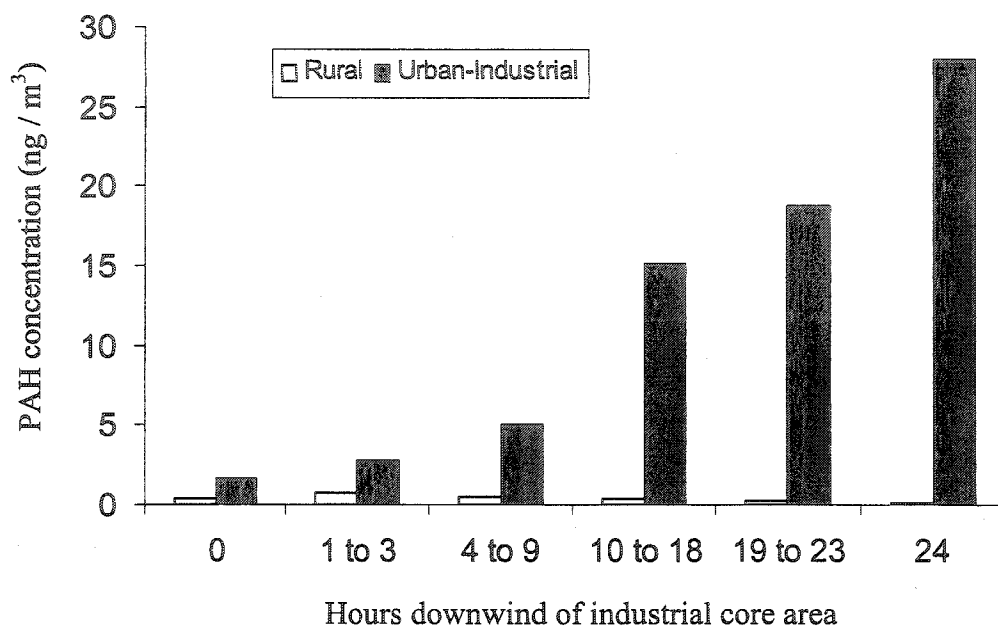


Fig. 2. Total PAH concentration measured in TSP samples grouped into categories based on the daily number of hours that sentinel mice at the urban-industrial site were located in a downwind direction from the industrial core area. Samples collected on the same dates at the rural site were similarly grouped for comparison. The number of samples comprising these measurements, and the percentage of exposure days with similar PAH exposure can be determined from Table 1. No error bars are displayed because each measurement presented is from a number of samples pooled within each category.

SUPPLEMENTARY MATERIAL

Materials and Methods

Environmental Exposures - **(A) AMBIENT AIR:** we exposed lab mice in situ simultaneously at two locations in southern Ontario, Canada (1) urban-industrial site – near the industrial core area containing two large integrated steel mills (1 and 4 km away to the west) and a major highway (1 km away to the east) on Hamilton Harbour (43° 15'N, 79° 51'W), a polluted industrial area on western Lake Ontario, and (2) rural site - a small farm 30 km away from the urban-industrial site in an area removed from point sources of air pollution. At each field site we housed 21 male and 21 female outbred, Swiss-Webster mice (Harlan, Indianapolis, IN, USA), 7 to 9 weeks old inside a modified utility shed that allowed ambient air flow, as described in Somers *et al.* (51). **(B) HEPA-FILTERED AIR:** concurrent to ambient air exposures, inside the shed at each site we housed 21 mice of each sex inside a 130 x 75 x 80-cm clear plexiglass chamber that received ambient air first passed through a high-efficiency particulate air filtration unit (True-HEPA, model 200-001, Bemis Manufacturing Co., Sheboygan Falls, WI, USA). HEPA units ran continuously over the exposure period at a draw rate of 65 cubic feet per minute, and filters at each site were replaced after 35 days. In situ environmental exposures lasted the 10 weeks from 25 April to 4 July 2000, during which all mice received mouse chow and bottled water from the same commercial sources.

Mouse Breeding - We held all mice in same-sex groups (5 animals per cage) during the exposure and for 9 weeks post-treatment. Breeding pairs were then assigned randomly within each group, and left together for 7 days. All males except 10 in each of the urban-industrial and rural ambient air treatment groups were then euthanized, and tail tissue sampled for DNA extraction. The remaining males were mated a second time to additional, unexposed females, and later euthanized and tail tissue sampled for DNA

extraction. We monitored females for date of delivery and litter size, and weighed all pups at 24, 48, 72, and 120 hours after birth. Females and pups were euthanized and tail tissue sampled for DNA extraction when the pups were 5 days old. All animal procedures were approved by the McMaster University Animal Research Ethics Board following the guidelines of the Canadian Council on Animal Care.

Genetic Analysis - The pedigree DNA profiling approach we used to detect heritable DNA mutations at ESTR loci Ms6-hm and Hm-2 has been described in detail elsewhere (S1). Parentage confirmation and further mutation analyses were also conducted using multilocus ESTR probe MMS10 (S2).

Air Analyses - We used high-volume air samplers to collect total suspended particulate matter (TSP) at each field site for simultaneous 24-hr periods on 25 of the 70 mouse exposure days. From detailed wind direction data for Hamilton (Ontario Ministry of the Environment), we grouped filters for analysis based on the total number of hours the mice at the urban-industrial site would have been downwind (westerly component to wind direction) of the industrial core area on each sampling day. Samples collected on the same dates at the rural site were similarly grouped for comparison between the two locations. This resulted in 6 categories of exposure day ranging from 0 to 24 hours downwind time. We quantified 26 PAHs (Table S4), molecular weight 202 to 302 g/mol, in both TSP samples and on the HEPA filters using gas chromatography linked to mass spectrometry (S3). More inclusive data on PAH levels are available from the authors upon request.

Statistical analyses – Body mass of adult mice was compared among groups using a one-way analysis of variance (ANOVA). A Scheffe post hoc test was applied to any significant outcome. Mutation rates were calculated as the number of mutant bands out of

the total number scored for all offspring in each treatment group (mutation rate per-offspring band), and 95% confidence intervals estimated from the Poisson distribution (S4). We also calculated the average proportion of mutant bands detected on a per-family basis in each group. Overall per-family mutation rates for ESTR single loci Ms6-hm + Hm-2 combined, and multilocus probe MMS10 were compared among groups using two separate one-way ANOVA tests (one for each probe type). Separate two-way ANOVA tests were used to compare paternal and maternal mutation rates by site and HEPA-filtration treatment. A single two-way ANOVA was used to compare paternal and maternal mutation rates in families where exposed males were mated to unexposed females. For these same families, we compared overall mutation rates for ESTR single loci Ms6-hm and Hm-2 combined, and multilocus probe MMS10 between groups using two-sample T-tests. For all statistical analyses based on per-family mutation rates, we arcsine-transformed the data using an improved angular transformation procedure (S5). Scheffe post hoc testing was applied following significant outcomes of ANOVA. We tested for an association between the number of hours downwind of steel mills and air quality measurements using Kendall's *Tau* Correlation, and relative PAH concentration between sites was compared using a Wilcoxon Signed-Rank Test.

Results

Animal exposure and breeding - Experimental treatment did not affect the body mass of male mice 24 hours post-exposure, which averaged 30.5 ± 2.9 g, 30.5 ± 2.1 g, 29.1 ± 2.0 g, and 30.9 ± 2.7 g for rural, rural HEPA, urban-industrial, and urban-industrial HEPA groups, respectively (ANOVA, $F_{3,80} = 2.29$, $P = 0.084$). In contrast, female mice exposed to ambient air at the rural site (25.5 ± 2.2 g) were significantly heavier than those exposed at the urban-industrial site to either ambient (23.4 ± 1.5 g) or HEPA filtered air (22.8 ± 1.4 g), and the rural HEPA group was intermediate (24.2 ± 1.7 g; ANOVA, $F_{3,80} = 7.83$,

$P = 0.0001$). The cause of the differences in mass among females is unknown; however, breeding success, litter size, and pup mass did not differ significantly among any of the treatment groups (data not shown). Litter sizes could not be precisely determined in a number of cases because of female cannibalism of pups; this confounded comparison among sites and treatment groups.

Detailed mutation analyses - Overall mutation frequencies and average per-family mutation rates determined using ESTR single locus probes Ms6-hm and Hm-2, and multilocus probe MMS10 are summarized in Table S1. Per-family mutation rates at ESTR single loci Ms6-hm and Hm-2 differed significantly among the rural, rural HEPA, urban-industrial, and urban-industrial HEPA groups (ANOVA, $F_{3,69} = 7.84$, $P = 0.0002$); post-hoc testing revealed that the urban-industrial group had higher mutation rates than any of the other three groups. MMS10 mutation rates also differed among the same four treatment groups (ANOVA, $F_{3,69} = 5.47$, $P = 0.0021$). The urban-industrial group had significantly higher MMS10 mutation rates than the rural HEPA group, and the urban-industrial HEPA and rural groups were intermediate. Both single and multilocus mutation rates differed between groups where males were exposed at either the rural or urban-industrial site and mated to unexposed females (T-test, $t = 2.71$, $df = 14$, $P = 0.0170$; $t = 2.18$, $df = 14$, $P = 0.0466$, for single and multilocus respectively).

Paternal and maternal mutation frequencies and average per-family mutation rates at ESTR single loci Ms6-hm and Hm-2 combined are given in Table S2. A summary of the two-way ANOVA comparing paternal and maternal mutation rates in exposed males mated with unexposed females is provided in Table S3.

Chemical analysis of air samples - Table S4 contains the list of 26 polycyclic aromatic hydrocarbons assayed for in this study. Evaluation of the protection efficiency of HEPA-filtration against a particular group of environmental contaminants under ambient conditions as we describe for PAHs in this study is rare. Factors contributing to uncertainty regarding this analysis are therefore not well established. We conservatively estimated based on comparing total PAH concentration in TSP to that on HEPA filters, that our filtration unit removed 55% to 61% of available PAHs at the urban-industrial site. This is likely an underestimate of the actual performance of the unit for several reasons. First, we restricted our analysis to the units' paper HEPA filters, and did not analyze PAH content in larger particles trapped on the plastic pre-filters. This may explain a portion of the difference between PAH levels in TSP samples and on the HEPA filters. Second, each HEPA-filter was in place for 35 days (50% of the mouse exposure), and drew close to 100 000 m³ of air. It is likely that some of the PAHs originally associated with particles trapped by the HEPA-filter became volatile, and were lost from the filter in a gaseous state. Sentinel animals would not have been protected from exposure to these PAHs in their volatile state.

Table S1. Detailed summary of germline ESTR mutation data in six groups of sentinel mice exposed to ambient and HEPA filtered air.

Group ^a	Probe ^b	Pups Scored ^c (litters)	Bands Scored ^c	Mutant Bands	Mutation Rate Per-Offspring Band (95% CI) ^d	Average Per-Family Mutation Rate ^e ±SD
Rural	Ms6-hm	99 (18)	186	19	0.102 (0.061, 0.160)	0.099 ± 0.071
	Hm-2	98 (18)	181	20	0.111 (0.067, 0.171)	
	Total		367	39	0.106 (0.076, 0.145)	
	Single					
	MMS10	99 (18)	1581	26	0.016 (0.011, 0.024)	
Rural HEPA	Ms6-hm	106 (18)	194	22	0.113 (0.071, 0.172)	0.136 ± 0.092
	Hm-2	100 (18)	192	26	0.135 (0.088, 0.199)	
	Total		386	48	0.124 (0.092, 0.165)	
	Single					
	MMS10	106 (18)	1553	18	0.012 (0.007, 0.018)	
Rural male	Ms6-hm	45 (8)	85	7	0.082 (0.033, 0.171)	0.075 ± 0.067
	Hm-2	45 (8)	83	6	0.072 (0.026, 0.158)	
	Total		168	13	0.077 (0.041, 0.133)	
	Single					
	MMS10	45 (8)	744	9	0.012 (0.006, 0.023)	
Urban-Industrial	Ms6-hm	113 (20)	212	56	0.264 (0.199, 0.343)	0.223 ± 0.068
	Hm-2	111 (20)	216	39	0.180 (0.128, 0.247)	
	Total		428	95	0.222 (0.179, 0.271)	
	Single					
	MMS10	114 (20)	1781	61	0.034 (0.026, 0.044)	
Urban-Industrial HEPA	Ms6-hm	98 (17)	182	23	0.126 (0.080, 0.189)	0.139 ± 0.102
	Hm-2	92 (16)	171	24	0.140 (0.089, 0.209)	
	Total		353	47	0.133 (0.098, 0.177)	
	Single					
	MMS10	96 (17)	1501	32	0.021 (0.015, 0.030)	
Urban-Industrial male	Ms6-hm	48 (8)	90	13	0.144 (0.077, 0.248)	0.187 ± 0.097
	Hm-2	46 (8)	86	21	0.244 (0.151, 0.374)	
	Total		176	34	0.193 (0.134, 0.270)	
	Single					
	MMS10	48 (8)	817	24	0.029 (0.019, 0.044)	

^a Groups labeled Rural male and Urban-Industrial male consist of pedigrees derived from male mice exposed to ambient air and then bred to unexposed females.

^b Mutation rates for ESTR multilocus probe MMS10 do not include Ms6-hm and Hm-2 bands co-detected with this marker.

^c In some instances single locus bands were too small to be detected, resulting in a reduction in the number of litters, pups or bands scored.

^d The 95% confidence intervals shown were estimated for the overall per-band mutation frequency in each group from the Poisson distribution. Note that these estimates are from pooled frequency data for all offspring in each group combined, and may therefore be somewhat narrower than expected.

^e The per-family average mutation rates are based on combined Ms6-hm and Hm-2 data for each litter for single locus probes, or MMS10 data only for each litter. Values are based on total numbers of mutations divided by the total number of bands scored in each family, and then averaged over the number of litters in each treatment group.

Table S2. Detailed summary of paternal and maternal ESTR mutation data. Footnotes are as listed in Table 1.

Group ^a	Bands Scored ^c	Mutant Bands	Mutation Rate Per-Offspring Band (95% CI) ^d	Average Per-Family Mutation Rate ^e $\pm$ SD
PATERNAL				
Rural	191	27	0.141 (0.093, 0.206)	0.142 $\pm$ 0.099
Rural HEPA	192	29	0.151 (0.101, 0.217)	0.164 $\pm$ 0.107
Rural male	79	8	0.101 (0.043, 0.200)	0.098 $\pm$ 0.121
Urban-Industrial	208	61	0.293 (0.224, 0.377)	0.301 $\pm$ 0.108
Urban-Industrial HEPA	177	25	0.141 (0.091, 0.209)	0.134 $\pm$ 0.079
Urban-Industrial male	85	24	0.282 (0.180, 0.421)	0.282 $\pm$ 0.117
MATERNAL				
Rural	176	12	0.068 (0.035, 0.119)	0.064 $\pm$ 0.073
Rural HEPA	194	19	0.098 (0.059, 0.153)	0.107 $\pm$ 0.134
Rural male	89	5	0.056 (0.018, 0.132)	0.054 $\pm$ 0.080
Urban-Industrial	220	34	0.154 (0.107, 0.216)	0.151 $\pm$ 0.120
Urban-Industrial HEPA	176	22	0.125 (0.078, 0.190)	0.132 $\pm$ 0.154
Urban-Industrial male	91	10	0.110 (0.052, 0.203)	0.104 $\pm$ 0.087

Table S3. Summary of two-way ANOVA results for the effect of environmental exposure on ESTR mutation rates in families where exposed males were mated to unexposed females.

Source	<i>df</i>	<i>F</i> -value	P
Exposure Site	1, 28	9.39	0.0048
Sex (male/female)	1, 28	8.41	0.0072
Interaction	1, 28	2.37	0.1347

Table S4. List of polycyclic aromatic hydrocarbons (PAH) measured in TSP and HEPA filter samples of particulate from *in situ* mouse exposures.

PAH Compound	Molecular Weight (g/mol)
Pyrene	202
Benzo[a]fluorene	216
Benzo[b]fluorene	216
Benzo[b]naphtho[2,1-d]thiophene	234
Benzo[ghi]fluoranthene	226
benzo[c]phenanthrene	228
Benzo[b]naphtho[2,3-d]thiophene	234
Benz[a]anthracene	228
Cyclopenta[cd]pyrene	226
Chrysene	228
Benzanthrone	230
Benz[a]anthracene-7,12-dione	258
Benzo[b]fluoranthene	252
Benzo[k]fluoranthene	252
Benzo[j]fluoranthene	252
Benzo[e]pyrene	252
Benzo[a]pyrene	252
Perylene	252
Indeno[1,2,3-cd]pyrene	276
Dibenz[a,c]anthracene	278
Picene	278
Benzo[ghi]perylene	276
Coronene	300
Dibenzo[a,e]pyrene	302
Dibenzo[a,i]pyrene	302
Dibenzo[a,h]pyrene	302

References and Notes:

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APPENDIX TO CHAPTER 3

The tables included in this section contain detailed measurements of the individual polycyclic aromatic hydrocarbons (PAH) comprising the totals summarised in the main portion of Chapter 3.

Table 1. Individual PAH measurements and percent composition of TSP collected at the Urban-Industrial mouse exposure site. Samples were grouped based on the daily number of hours that the sampler was downwind of the industrial core area.

Daily Time Downwind		0 hrs		1 - 3 hrs		4 - 9 hrs		10 - 18 hrs		19 - 23 hrs		24 hrs	
Compound	MW	(pg/m3)	% Comp	(pg/m3)	% Comp	(pg/m3)	% Comp	(pg/m3)	% Comp	(pg/m3)	% Comp	(pg/m3)	% Comp
Pyrene	202	138.5	8.3	175.1	6.4	226.3	4.4	846.9	5.6	838.7	4.5	1132.3	4.0
Benzo[a]fluorene	216	13.8	0.8	16.8	0.6	33	0.6	100.9	0.7	107.6	0.6	146	0.5
Benzo[b]fluorene	216	8.2	0.5	12.7	0.5	26.3	0.5	71.8	0.5	69.3	0.4	100.1	0.4
Benzo[b]naphtho[2,1-d]thiophene	234	23.8	1.4	32.5	1.2	61.5	1.2	157.4	1.0	177.3	0.9	196.6	0.7
Benzo[ghi]fluoranthene	226	27.2	1.6	29.1	1.1	35.6	0.7	143.2	0.9	147.3	0.8	173.1	0.6
benzo[c]phenanthrene	228	10.1	0.6	15.1	0.6	20.6	0.4	60.5	0.4	90.4	0.5	91.5	0.3
Benzo[b]naphtho[2,3-d]thiophene	234	6.4	0.4	9.4	0.3	19.1	0.4	86.6	0.6	71.6	0.4	78.4	0.3
Benz[a]anthracene	228	77	4.6	117.5	4.3	257.3	5.1	689.6	4.6	706.2	3.8	1093.2	3.9
Cyclopenta[cd]pyrene	226	7.4	0.4	12.7	0.5	11	0.2	82.7	0.5	113	0.6	206.6	0.7
Chrysene	228	297.7	17.9	376.4	13.8	744.6	14.6	1678.8	11.1	1967.9	10.5	2687.5	9.6
Benzanthrone	230	47.5	2.9	60.7	2.2	70.9	1.4	210.4	1.4	422	2.3	394.6	1.4
Benz[a]anthracene-7,12 dione	258	22.3	1.3	45.7	1.7	76.8	1.5	132.5	0.9	220.6	1.2	310.3	1.1
Benzo[b]fluoranthene	252	236.6	14.2	416.1	15.2	815.7	16.0	2129.1	14.1	2877.6	15.4	4445.5	15.9
Benzo[k]fluoranthene	252	82.4	5.0	182.6	6.7	392.8	7.7	1054.6	7.0	1294.4	6.9	2101.7	7.5
Benzo[j]fluoranthene	252	83.9	5.0	166.2	6.1	352.5	6.9	920.6	6.1	1239.1	6.6	1901.8	6.8
Benzo[e]pyrene	252	151.3	9.1	259.7	9.5	532.8	10.5	1513.5	10.0	2126.2	11.3	3289.7	11.7
Benzo[a]pyrene	252	93.9	5.6	184.8	6.8	434.3	8.5	1407.9	9.3	1395.6	7.4	2717.7	9.7
Perylene	252	24.2	1.5	54	2.0	127.1	2.5	410.8	2.7	441	2.4	815.5	2.9
Indeno[1,2,3-cd]pyrene	276	119.7	7.2	225.5	8.2	341.2	6.7	1383.6	9.2	1782.6	9.5	2938	10.5
Dibenz[a,c]anthracene	278	10.7	0.6	27.2	1.0	42.3	0.8	201.6	1.3	213.5	1.1	278.7	1.0
Picene	278	12.3	0.7	29.5	1.1	41	0.8	151	1.0	198.1	1.1	258.1	0.9
Benzo[ghi]perylene	276	105.8	6.4	196.9	7.2	292.4	5.7	1074.9	7.1	1513.3	8.1	1663.2	5.9
Coronene	300	43	2.6	66.4	2.4	76	1.5	354.2	2.3	416.7	2.2	481.8	1.7
Dibenzo[a,e]pyrene	302	11.4	0.7	22.6	0.8	32.1	0.6	184.2	1.2	183.8	1.0	245.4	0.9
Dibenzo[a,i]pyrene	302	8.8	0.5	1.9	0.1	25.2	0.5	69.1	0.5	131.8	0.7	248.1	0.9
Dibenzo[a,h]pyrene	302	0	0.0	0	0.0	0	0.0	3.6	0.0	0	0.0	3.6	0.0
Total PAH		1663		2737		5088		15120		18745		27999	

Table 2. Individual PAH measurements and percent composition of TSP collected at the Rural mouse exposure site. Samples were grouped to match dates analysed at the urban-industrial site in Table 1.

Daily Time Downwind		0 hrs		1 - 3 hrs		4 - 9 hrs		10 - 18 hrs		19 - 23 hrs		24 hrs	
Compound	MW	(pg/m ³)	% comp	(pg/m ³)	% comp	(pg/m ³)	% comp	(pg/m ³)	% comp	(pg/m ³)	% comp	(pg/m ³)	% comp
Pyrene	202	35.5	8.2	54.1	6.9	35.7	6.8	28.1	7.4	19.4	7.8	18.3	11.2
Benzo[a]fluorene	216	3.4	0.8	4.9	0.6	3.8	0.7	2.5	0.7	1.4	0.6	1.3	0.8
Benzo[b]fluorene	216	2.3	0.5	3.1	0.4	2.5	0.5	1.7	0.4	1	0.4	0.7	0.4
Benzo[b]naphtho[2,1-d]thiophene	234	5.1	1.2	8.7	1.1	6.9	1.3	4.4	1.2	2.5	1.0	2.2	1.3
Benzo[ghi]fluoranthene	226	6.1	1.4	8.5	1.1	7.1	1.3	4.9	1.3	4.8	1.9	2.8	1.7
benzo[c]phenanthrene	228	2.4	0.6	3.8	0.5	3.4	0.6	2.1	0.6	1.1	0.4	0.9	0.5
Benzo[b]naphtho[2,3-d]thiophene	234	1.7	0.4	3.2	0.4	4.1	0.8	1.6	0.4	1.1	0.4	0.7	0.4
Benz[a]anthracene	228	11.3	2.6	18.6	2.4	16.7	3.2	13.7	3.6	6.2	2.5	5.4	3.3
Cyclopenta[cd]pyrene	226	1.8	0.4	3.5	0.4	3.3	0.6	2	0.5	1.3	0.5	0.8	0.5
Chrysene	228	48.8	11.3	90.2	11.4	46.4	8.8	44.9	11.8	30.9	12.4	22.8	13.9
Benzanthrone	230	0.0	0.0	29.6	3.8	16.7	3.2	12.3	3.2	19.1	7.7	8.8	5.4
Benz[a]anthracene-7,12 dione	258	6.8	1.6	15.7	2.0	6.8	1.3	5.4	1.4	7.7	3.1	2.6	1.6
Benzo[b]fluoranthene	252	72.1	16.7	123.1	15.6	69.2	13.2	55.1	14.5	33.6	13.5	19	11.6
Benzo[k]fluoranthene	252	30.7	7.1	44.3	5.6	34.6	6.6	23.2	6.1	13.2	5.3	8.5	5.2
Benzo[j]fluoranthene	252	29.5	6.9	42.5	5.4	33.5	6.4	23	6.0	15.4	6.2	8.7	5.3
Benzo[e]pyrene	252	49.5	11.5	81.9	10.4	52.3	9.9	36.6	9.6	23.1	9.3	13.1	8.0
Benzo[a]pyrene	252	28.8	6.7	45.5	5.8	38.2	7.3	28	7.3	12.2	4.9	9.3	5.7
Perylene	252	4.2	1.0	9.6	1.2	9.6	1.8	5.4	1.4	2.8	1.1	1.7	1.0
Indeno[1,2,3-cd]pyrene	276	49.1	11.4	85.3	10.8	51.1	9.7	34.6	9.1	22.2	8.9	13.1	8.0
Dibenz[a,c]anthracene	278	2.4	0.6	6.4	0.8	7.2	1.4	2.8	0.7	2.1	0.8	0.9	0.5
Picene	278	5.8	1.3	9.7	1.2	6.8	1.3	4.4	1.2	2.8	1.1	1.5	0.9
Benzo[ghi]perylene	276	33.9	7.9	65.2	8.3	39.8	7.6	27.2	7.1	19.1	7.7	13.8	8.4
Coronene	300	0.0	0.0	23.8	3.0	21	4.0	11.4	3.0	3.6	1.4	6	3.7
Dibenzo[a,e]pyrene	302	0.0	0.0	6.9	0.9	6.8	1.3	3.8	1.0	1.8	0.7	1.1	0.7
Dibenzo[a,i]pyrene	302	0.0	0.0	0	0.0	2.7	0.5	1.9	0.5	0.9	0.4	0	0.0
Dibenzo[a,h]pyrene	302	0.0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
Total PAH		431.2		788.1		526.2		381.0		249.3		164.0	

Table 3. Average overall percent composition of individual PAH compounds measured in TSP at the two field sites.

Compound	Freelton	Hamilton
Pyrene	8.0	5.5
Benzo[a]fluorene	0.7	0.6
Benzo[b]fluorene	0.4	0.4
Benzo[b]naphtho[2,1-d]thiophene	1.2	1.1
Benzo[ghi]fluoranthene	1.5	1.0
benzo[c]phenanthrene	0.5	0.5
Benzo[b]naphtho[2,3-d]thiophene	0.5	0.4
Benz[a]anthracene	2.9	4.4
Cyclopenta[cd]pyrene	0.5	0.5
Chrysene	11.6	12.9
Benzanthrone	3.9	1.9
Benz[a]anthracene-7,12 dione	1.8	1.3
Benzo[b]fluoranthene	14.2	15.1
Benzo[k]fluoranthene	6.0	6.8
Benzo[j]fluoranthene	6.0	6.3
Benzo[e]pyrene	9.8	10.4
Benzo[a]pyrene	6.3	7.9
Perylene	1.3	2.3
Indeno[1,2,3-cd]pyrene	9.6	8.5
Dibenz[a,c]anthracene	0.8	1.0
Picene	1.2	0.9
Benzo[ghi]perylene	7.8	6.7
Coronene	2.5	2.1
Dibenzo[a,e]pyrene	0.8	0.9
Dibenzo[a,i]pyrene	0.2	0.5
Dibenzo[a,h]pyrene	0.0	0.0

Table 4. Detailed PAH data for HEPA filter extractions at each site. Time period a = April 25 to May 30 / 2000, and period b = May 31 to July 4 / 2000. We also extracted a blank (unused) filter, which contained trace amounts of pyrene (96.3 pg/m³ of air drawn), but no other PAHs.

Compound	MW	Pier 25a		Pier 25b		Freelton a		Freelton b	
		Pg/m3	% comp	Pg/m3	% comp	Pg/m3	% comp	Pg/m3	% comp
Pyrene	202	526.9	7.4	443.1	5.2	22.9	13.4	14.0	25.2
Benzo[a]fluorene	216	91.5	1.3	69.0	0.8	0.0	0.0	0.0	0.0
Benzo[b]fluorene	216	80.7	1.1	61.6	0.7	0.0	0.0	0.0	0.0
Benzo[b]naphtho[2,1-d]thiophene	234	132.7	1.9	117.0	1.4	0.0	0.0	0.0	0.0
Benzo[ghi]fluoranthene	226	74.4	1.0	88.5	1.0	0.0	0.0	0.0	0.0
benzo[c]phenanthrene	228	62.8	0.9	53.4	0.6	0.0	0.0	0.0	0.0
Benzo[b]naphtho[2,3-d]thiophene	234	49.0	0.7	77.8	0.9	0.0	0.0	0.0	0.0
Benz[a]anthracene	228	217.5	3.1	169.1	2.0	0.0	0.0	0.0	0.0
Cyclopenta[cd]pyrene	226	46.9	0.7	48.0	0.6	0.0	0.0	0.0	0.0
Chrysene	228	1082.6	15.2	1505.7	17.8	16.9	9.9	0.0	0.0
Benzanthrone	230	109.1	1.5	278.0	3.3	0.0	0.0	0.0	0.0
Benzo[a]anthracene-7,12 dione	258	117.0	1.6	221.3	2.6	0.0	0.0	0.0	0.0
Benzo[b]fluoranthene	252	995.5	14.0	1301.4	15.4	42.5	24.8	5.0	9.1
Benzo[k]fluoranthene	252	637.1	9.0	700.9	8.3	15.2	8.8	9.1	16.3
Benzo[j]fluoranthene	252	493.2	6.9	481.8	5.7	17.6	10.2	0.0	0.0
Benzo[e]pyrene	252	623.7	8.8	860.3	10.2	24.1	14.1	17.2	30.9
Benzo[a]pyrene	252	532.9	7.5	635.1	7.5	13.1	7.6	10.4	18.6
Perylene	252	160.7	2.3	303.2	3.6	0.0	0.0	0.0	0.0
Indeno[1,2,3-cd]pyrene	276	553.5	7.8	679.5	8.0	0.0	0.0	0.0	0.0
Dibenz[a,c]anthracene	278	37.1	0.5	80.8	1.0	0.0	0.0	0.0	0.0
Picene	278	49.0	0.7	24.6	0.3	0.0	0.0	0.0	0.0
Benzo[ghi]perylene	276	433.1	6.1	266.2	3.1	19.3	11.3	0.0	0.0
Coronene	300	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Dibenzo[a,e]pyrene	302	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Dibenzo[a,i]pyrene	302	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Dibenzo[a,h]pyrene	302	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
TOTAL PAH		7106.9		8466.4		171.5		55.6	

CHAPTER 4

An Approach to Feeding High Percentage Fish Diets to Mice for Human and Wildlife
Toxicology Studies

Christopher M. Somers¹, Eduardo V. Valdes², James S. Quinn¹

- 1) Department of Biology, McMaster University, 1280 Main St. West, Hamilton,
ON, Canada, L8S 4K1
- 2) Nutrition Centre, Metropolitan Toronto Zoo, 361A Old Finch Ave, Scarborough,
ON, Canada, M1B 5K7

ABSTRACT

Experimental feeding of sport fish to rodents has been an important tool for studying biological effects induced by a contaminated fish diet. Most rodent feeding studies have used relatively low levels of tissue from large fish species incorporated into diets fed to rats, and have given little consideration to issues of diet palatability or nutrition. There are currently no rodent diet models suitable for assessing risk to human populations with diets very high in daily fish content, or wildlife species consuming high percentages of whole, small-bodied fish. In this study we describe an approach to feeding mice high percentages of homogenized, whole fish using Atlantic herring (*Clupea harengus*) as a test species. We created a novel gel diet medium for mice that contains a variety of nutritional supplements, and is flexible in terms of the fish percentage that can be incorporated. In choice trials, mice preferred 30% and 35% fish gels to their regular commercial dry chow, indicating that the gel diet medium was palatable. In a longer feeding trial, mice ate 35% fish gel for 12 days and 50% fish gel for 12 days (total of 24 consecutive days), and did not differ in body mass compared to age and sex-matched controls. We conclude that our fish-based gel diet is suitable for rodent feeding trials in toxicology studies examining dose responses to fish consumption, and risk in human and wildlife populations where daily fish intake is high. Our general approach may also be applicable for feeding mice materials other than fish.

INTRODUCTION

The consumption of sport fish from certain areas, such as the Laurentian Great Lakes of North America, has been identified as a potentially important source of human

exposure to a suite of environmental contaminants (Kearney *et al.* 1999, Schantz *et al.* 1999, Nadon *et al.* 2002, Cole *et al.* 2002). A number of health effects potentially associated with contaminant exposure have been documented in sport fish consumers, including reproductive problems (Courval *et al.* 1999, Buck *et al.* 2000), neuro-muscular impairments (Schantz *et al.* 1999, Schantz *et al.* 2001), and behavioral irregularities in children whose mothers eat fish during pregnancy (Lonky *et al.* 1996, Schell *et al.* 2001). Due to a variety of possible confounding factors in human studies, however, it has been difficult to establish strong causal links between sport fish consumption and particular health effects.

To overcome the difficulties inherent in human studies, rodents fed fish-based diets have been used as a more controlled experimental system to examine biological responses to contaminated fish consumption. These studies have identified a variety of negative effects, including decreased growth rates (Sonstegard and Leatherland 1978), increased thyroid gland size and structural changes (Sonstegard and Leatherland 1979, Leatherland and Sonstegard 1980), accumulation of organo-chlorine contaminants in body tissues (Feeley and Jordan 1998, Jordan and Feeley 1999, Stewart *et al.* 2000), immunological changes (Tryphonas *et al.* 1998), and behavioural alterations (Gerstenberger *et al.* 2000, Stewart *et al.* 2000). However, not all recent rodent feeding experiments have shown significant or adverse effects (e.g., Beattie *et al.* 1996, Arnold *et al.* 1998), and the position of some regulatory agencies is that the risk to anglers from sport fish is minimal as long as consumption advisories are followed (Feeley *et al.* 1998).

In some situations fish consumption may still pose a health risk, but advisories do not apply or cannot be followed. For example, sport fish advisories do not generally

apply to aquaculture-raised salmon even though they can have relatively high tissue contaminant levels (Hites *et al.* 2004), and socio-economic constraints or cultural traditions may not permit a reduction in subsistence fish consumption in certain human populations (e.g., Frery *et al.* 2001, Dorea *et al.* 2004). In addition, piscivorous wildlife species do not have the ability to make a choice about whether to eat fish. The high body burden of contaminants previously measured in predatory sport fish and birds (e.g. Ewins *et al.* 1994, Stewart *et al.* 2000) in the Great Lakes is almost certainly due to cumulative exposure through a diet of small-bodied fish, yet wildlife health effects caused by consumption of small fish species have not been considered in rodent feeding studies.

There is currently a need to expand the application of rodent feeding experiments to assess toxicological risk for fish consumers beyond recreational anglers, but suitable methods have not been established. A review of published rodent feeding studies (Table 1) reveals a lack of diversity in fish species tested, with the majority of experiments assessing the effects of Great Lakes salmon fed to rats. In addition, there has been little consistency in the methods used for fish-diet preparation or dietary levels of fish delivered to rodents, and most studies have not addressed animal welfare issues such as palatability or nutritional supplementation of fish-based diets. The purpose of this study was to develop and test a high fish content diet for mice for use in toxicology studies. We describe a new approach to feeding whole, homogenized fish to mice using a novel gel diet medium. Our approach provides flexibility in the amount of fish that can be incorporated into the diet, allowing high fish levels while continuing to provide basic nutritional components to mice in a palatable format.

METHODS

Diet Preparation – The fish used in this study were commercially purchased Atlantic herring (*Clupea harengus*) that were on average ($\pm$ SD) 27.8 ± 3.0 cm long and weighed 175 ± 60.5 g. Whole fish were coarsely ground with a Hobart 4346 mixer-grinder (Hobart Manufacturing, Troy, Ohio), followed by fine homogenization into a smooth paste with a Hobart VCM-25 vertical cutter-mixer. Fish homogenate was combined with other ingredients and boiling water (Table 2), and mixed thoroughly with the VCM-25 to provide 3 separate diets consisting of either 30%, 35%, or 50% fish by weight. Each diet medium was poured into large plastic containers and allowed to set into a solid gel at 4°C for 6 hours. We cut the gel into 250g portions, which were sealed in plastic and stored at –20°C until the day prior to use. Each gel portion was thawed at 4°C for 24 hours before being delivered to mice.

Choice Trial – To determine the palatability of the fish-based diet, we provided 6 male mice, 6 to 8 weeks old (out-bred Swiss-Webster strain, Charles River Labs) with a pre-weighed excess of their normal commercial dry chow (Harlan Teklad 22/5), and a pre-weighed, smaller amount of either the 30% or 35% fish-based gel (in separate trials). Food was delivered to mice (1 or 2 animals per cage) in two small glass dishes on the floor of their cage at 17:00, and removed at 08:30 the next day. Choice trials were performed separately for 30% and 35% fish-based gels on two consecutive days. The 35% trial was extended for two additional days, during which we provided less commercial dry chow and more fish diet medium. We estimated consumption of each

food type based on the amount remaining in cages each morning, and weighed mice before and after the choice trial. For comparison, we monitored consumption and body mass in a control group of 5 male mice given pre-weighed quantities of commercial dry chow delivered in the same manner. All mice received tap water on an *ad lib* basis.

Feeding Trial – We returned all mice from the choice trial to an *ad lib* diet of commercial dry chow for two weeks. Six randomly selected individuals were then habituated to a fish-based diet by first providing them with an excess of commercial dry chow and a small amount of fish gel. We then decreased the amount of dry chow and increased the amount of fish gel provided each day for 5 days until no dry chow was added. We continued to deliver mice pre-weighed quantities of the 35% fish gel for 12 days, followed by 50% fish gel for 12 days. This resulted in consumption of a diet consisting entirely of fish-based gels for a total of 24 consecutive days. As in the choice trial, pre-weighed portions of food were delivered to mice (1 or 2 animals per cage) in two small glass dishes on the bottom of each cage at 17:00, and removed at 08:30 the next day. We monitored food consumption daily, and the body mass of each mouse approximately every 2 days. To estimate consumption, we weighed the amount of food remaining in each cage. Measurements of commercial chow were considered to be dry weight, whereas for the fish gels we present consumption data based on unadjusted wet weights of food consumed, as well as estimated dry weights based on 53% moisture lost during overnight periods. The conversion to dry weight for fish gels was determined by placing pre-weighed quantities of gel in glass dishes inside the animal room at 17:00, and weighing them at 08:30 the next day. For comparison, we fed a control group of 5 male

mice pre-weighed portions of commercial dry chow delivered in the same manner as the fish-based diet. We compared body mass between the two treatment groups over the course of the feeding trial. All mice received tap water on an *ad lib* basis.

Statistical Analyses - For the choice trial, we compared the mass of commercial dry chow consumed per mouse in the control and the fish-diet groups on each day using a two-sample t-test. The same test was used to compare mouse body masses before and after the choice trial. For the feeding trial we compared daily mass of food consumed between the fish-diet and control groups using a two-way repeated measures analysis of variance (RM-ANOVA). Within groups, we used RM-ANOVA to compare daily per-mouse consumption on days 1 through 12 vs days 13 through 24 of the feeding trial (a separate ANOVA for each group). This test was designed to determine whether there was any effect of switching from a 35% to a 50% fish gel. We compared mouse body mass between groups over the 24 days of the feeding trial using two-way RM-ANOVA. The Huynh-Feldt correction was applied to the degrees of freedom in all RM-ANOVA when the assumption of sphericity was not met.

RESULTS

Choice Trial – When mice in the fish-diet group were offered an excess of commercial dry chow and a small amount of 30% fish gel, they consumed approximately 42% less dry chow than their control counterparts (Fig 1a; t-test, $t = 6.14$, $df = 6$, $P = 0.0009$), and 100% of the fish gel provided. On day 2, mice in the fish-diet group consumed 33% less dry chow than those in the control group (Fig 1b; $t = 4.21$, $df = 6$, $P = 0.0056$), and nearly

all of the 35% fish gel provided. On day 3 mice in the fish-diet group were provided with an approximately equal amount of fish-gel and dry chow, with neither food medium individually providing enough mass to equal consumption by individuals in the control group. Under these conditions mice in the fish-diet group did not eat 100% of the available dry chow, continuing to consume less of it than their control counterparts (Fig 1c; $t = 2.74$, $df = 6$, $P = 0.0338$). Our findings were qualitatively similar on day 4, but mice in the fish-diet group consumed nearly all of the dry chow provided ($t = 1.05$, $df = 6$, $P = 0.3328$ for comparison to control), and proportionately less of the 35% fish gel than on any previous day of the choice trial. Body mass of mice did not differ between the control ($30.7\text{g} \pm 1.9$) and fish-diet ($30.6\text{g} \pm 1.2$) groups before beginning the choice trial (T-test, $t = 0.15$, $df = 9$, $P = 0.8854$), and no differences in body mass developed between groups during the 4-day choice trial ($29.0\text{g} \pm 1.77$, $30.2\text{g} \pm 1.18$ for control and fish-diet groups, respectively; $t = 1.34$, $df = 9$, $P = 0.2122$).

Feeding Trial – Mice in the control group consistently consumed between 4 and 5 grams of commercial dry chow on average each day throughout the 24-day trial (Fig. 2). When uncorrected for moisture loss, mice in the fish-diet group consumed between 8 and 10 grams of food on average each day. Using a correction factor of 53% moisture loss, mice in the fish-diet group actually consumed 4.5 to 6 grams of food each day, which on average was approximately 13% more than the control group (Fig. 2; RM-ANOVA, $F_{1,7} = 37.4$, $P < 0.001$). Within treatment groups, mice consumed significantly more of the 35% fish gel ($5.46\text{g} \pm 0.38$) on days 1 through 12 than the 50% fish gel ($4.75\text{g} \pm 0.40$) on days 13 through 24 (Fig. 2; RM-ANOVA, $F_{1,4} = 24.4$, $P = 0.008$). However, when the

control group was divided into the same two periods, consumption of dry chow was marginally higher on days 1 through 13 ($4.59\text{g} \pm 0.40$) than on days 14 through 24 ($4.26\text{g} \pm 0.26$), highlighting a general trend for mice to eat smaller quantities in the second half of the feeding trial (RM-ANOVA, $F_{1,3} = 9.45$, $P = 0.054$). Two-way RM-ANOVA revealed that trial day explained a significant proportion of the variance in mouse body mass, but diet treatment did not (Fig 3; Table 3). In addition, there was a significant interaction between trial day and diet treatment, highlighting the switch from slightly larger body mass in the control group in the first half of the trial, to slightly larger body mass in the fish-diet group beginning on day-15 (Fig. 3).

DISCUSSION

On days 1 and 2 of the choice trial mice in the fish-diet group ate nearly all of the 30% and 35% fish-based gels despite an available excess of commercial dry chow. This suggests that when given the choice, they actually preferred to eat the fish gels. In addition, on days 3 and 4 when approximately equal quantities of dry chow and 35% fish gel were offered, mice did not eat 100% of the dry chow provided even though it was less than the amount consumed by individuals in the control group. This demonstrates that mice were not simply eating as much dry chow as possible and then turning to the fish gel to supplement their daily intake requirements, but rather eating both food types interchangeably. These findings were qualitatively supported by behavioural observations showing that when both dry chow and fish gel were available in the same dish, mice would often begin eating the fish gel first. We conclude based on the outcome of the choice trial that both 30% and 35% fish-based gel diets were palatable for mice, and that

these high dietary levels of fish do not present behavioural obstacles for conducting toxicology studies, such as taste or smell aversion.

In the longer feeding trial, mice were able to consume a diet containing high fish percentages for 24 days without changes in body mass compared to an age and sex-matched control group. Daily mass of food consumed differed between treatment groups, but this finding should be interpreted with caution because of uncertainty regarding the moisture loss conversion for fish gels. Qualitatively, coat condition and behaviour of mice in the fish diet group did not appear to differ from that of the control animals at any point during the trial. Daily consumption of the 50% fish gel on days 13 through 24 was significantly lower than for the 35% gel on days 1 through 12, which initially suggested that the 50% gel might be less palatable. However, given that body mass of the mice in the fish-diet group did not decrease relative to the control group during days 13 through 24, it is more likely that the 50% fish gel had a higher mass-specific energy content, and that lower consumption was sufficient to meet daily intake requirements. In addition, daily consumption of dry chow in the control group also tended to decrease in the second half of the trial, suggesting that reduced consumption in both groups was independent of diet treatment. Given that the mice were habituated to being housed alone or in pairs, we suggest that they may have responded initially with higher consumption at the onset of the feeding trial because of the sudden restriction in food availability following a long period of *ad lib* feeding. We conclude from the feeding trial that mice were not adversely affected by a diet very high in fish content over a 24-day test period.

No other studies that we are aware of have formally reported on the palatability of rodent diets that have been modified to include fish tissue. Of the published examples

reviewed here (Table 1), only two papers make mention of preliminary palatability testing prior to designing their experiments. Villeneuve *et al.* (1981) reported that levels of up to 20% salmon by wet weight did not affect palatability to rats, but they do not describe how testing was conducted, or how this conclusion was reached. Similarly, Chu *et al.* (1984) report that levels of freeze-dried salmon up to 5.8% did not affect palatability for rats, but no details on the testing methods are provided. We propose that the palatability of fish-based diets may be important for rodent feeding studies for two reasons. First, smell or taste aversion to a diet in the absence of an alternative food source may cause delayed or reduced consumption and ultimately stress in experimental animals. This is intuitive, and it is somewhat surprising that a number of published studies provide no information on consumption during their experiments (e.g., Sonstegard and Leatherland 1978, 1979, Leatherland and Sonstegard 1980, Cleland *et al.* 1987, Cleland *et al.* 1988, Hertzler 1990, Daly 1991, Beattie *et al.* 1996, Daly 1998, Stewart *et al.* 2000). This information would be especially important in experiments that do not include reference fish diets (e.g., Gerstenberger *et al.* 2000), or that compare the effects of diets based on different fish species in the absence of a commercial diet control (e.g., Beattie *et al.* 1996). Second, for ethical reasons there is an increasing demand on researchers to take all steps possible to minimise the negative experience of animals during experimental procedures. If a palatable diet formulation is available, it should be used over a less palatable alternative. Given the demonstrated preference of mice for our fish-based gel over their commercial dry chow, our approach addresses both of the above concerns.

It is also important to recognise that diets high in fish content are abnormal for laboratory rodents and may not be nutritionally adequate for long-term feeding trials. Previous studies have been inconsistent in the approach used to address nutritional concerns. In early experiments rats were provided with 100% ground salmon only, with no nutritional adjustment (Sonstegard and Leatherland 1978, 1979, Leatherland Sonstegard 1980). Some later studies simply added fish material to commercial rodent chow (e.g., Villeneuve et al. 1981, Chu et al. 1984, Daly et al. 1991), whereas others have taken elaborate steps to balance protein, fatty acid, phosphorous, and calcium content (Arnold et al. 1998), or have provided extensive vitamin and mineral supplements (Cleland et al. 1987, 1988). Some biological endpoints monitored in rodent feeding studies, such as growth rates, and organ mass or cellular structure may be affected directly by nutritional deficiencies independent of contaminants in the diet. This suggests that at least some form of nutritional supplementation is necessary for long-term experiments, and that body mass and/or growth rate comparisons among treatment groups should be reported for any rodent feeding trial. In our diet formulation we included two types of commercial chow with different nutritional profiles. We also supplemented gels with a vitamin mixture, mono dical phosphate, thiamine, and vitamin E. With this recipe, we were able to deliver mice 35 to 50% fish for twenty four days with no effect on body mass, suggesting that it is a nutritionally suitable diet for longer term studies.

Gels containing homogenised food items have been used extensively for feeding a variety of zoo animals (Oyarzun and Santor 1980, Molitoris et al. 1998). The major advantage of this approach is the flexibility it allows in terms of what food items can be delivered to animals, their quantity, and the option to include nutritional supplements.

This approach has not been applied previously in toxicology studies, but offers the same advantages, particularly for delivering food items to laboratory animals that are unusual or potentially unpalatable. We have successfully created and tested a fish-based gel diet for feeding homogenised, whole fish to mice for human and wildlife toxicology studies. We conclude based on our pilot study that this approach offers a great deal of promise, particularly for testing biological effects induced by diets very high in fish content, and have since applied the technique to a much larger study using wild-caught fish (Somers et al., *in prep*).

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Table 1. Summary of rodent feeding studies. Palatability and Nutrition columns indicate whether data on these topics were reported.

Reference	Rodent	Fish Species	Dietary %	Delivery	Palatability	Nutrition	Endpoint
Sonstegard and Leatherland (1978)	Rat	Salmon	100	Fresh	N	N	Growth
Sonstegard and Leatherland (1979)	Rat	Salmon	100	Fresh	N	N	Thyroid size/structure
Leatherland and Sonstegard (1980)	Rat	Salmon	100	Fresh	N	N	Thyroid size/structure
Villeneuve et al. (1981)	Rat	Salmon	1.45 to 5.8	Freeze-dried	Y	N	Biochemistry/ histology
Chu et al. (1984)	Rat	Salmon	1.45 to 5.8	Freeze-dried	Y	N	Biochemistry/ histology
Cleland et al. (1987)	Mouse	Salmon	33	Freeze-dried	N	Y	Biochemistry/ histology
Cleland et al. (1988)	Mouse	Salmon	33	Freeze-dried	N	Y	Bioaccumulation
Hertzler (1990)	Rat	Salmon	8 to 30	Fresh	N	N	Behaviour
Daly (1991)	Rat	Salmon	30	Fresh	N	N	Behaviour
Beattie et al. (1996)	Rat	Lake trout, walleye, whitefish, carp	30	Fresh	N	N	Behaviour
Tryphonas et al. (1998)	Rat	Salmon	5 to 20	Freeze-dried	N	Y	Immune system
Arnold et al. (1998)	Rat	Salmon	5 to 20	Freeze-dried	N	Y	Reproduction
Feeley and Jordan (1998)	Rat	Salmon	5 to 20	Freeze-dried	N	Y	Bioaccumulation
Daly et al. (1998)	Rat	Salmon	30	Fresh	N	N	Behaviour
Jordan and Feeley (1999)	Rat	Salmon	5 to 20	Freeze-dried	N	Y	Bioaccumulation
Stewart et al. (2000)	Rat	Salmon	30	Fresh	N	N	Bioaccumulation
Gerstenberger et al. (2000)	Rat	Lake trout	30	Fresh	N	N	Endocrine system

Table 2. Ingredients in gel diet medium used to feed whole homogenized fish to mice. Amounts are given as a percentage of total wet mass.

Ingredient	Percentage of Total
Homogenized Atlantic herring	30% - 50%
Chick chow ^a	12.5%
Ground mouse chow ^b	12.5%
Unflavored gelatin	7%
TZ carnivore supplement ^c	2.5%
Mono dical phosphate	1.5%
Vitamin E	0.25%
Thiamin	0.025%
Distilled water ^d	13.7 - 33.7%

a – Purina Mills chick chow SG5065

b – Purina Mills mouse chow 9F5020

c – Toronto Zoo Carnivore Supplement (Roche Ltd)

d – distilled water was added to the diet preparation at 100°C; the amount of water added was adjusted to allow incorporation of more or less fish.

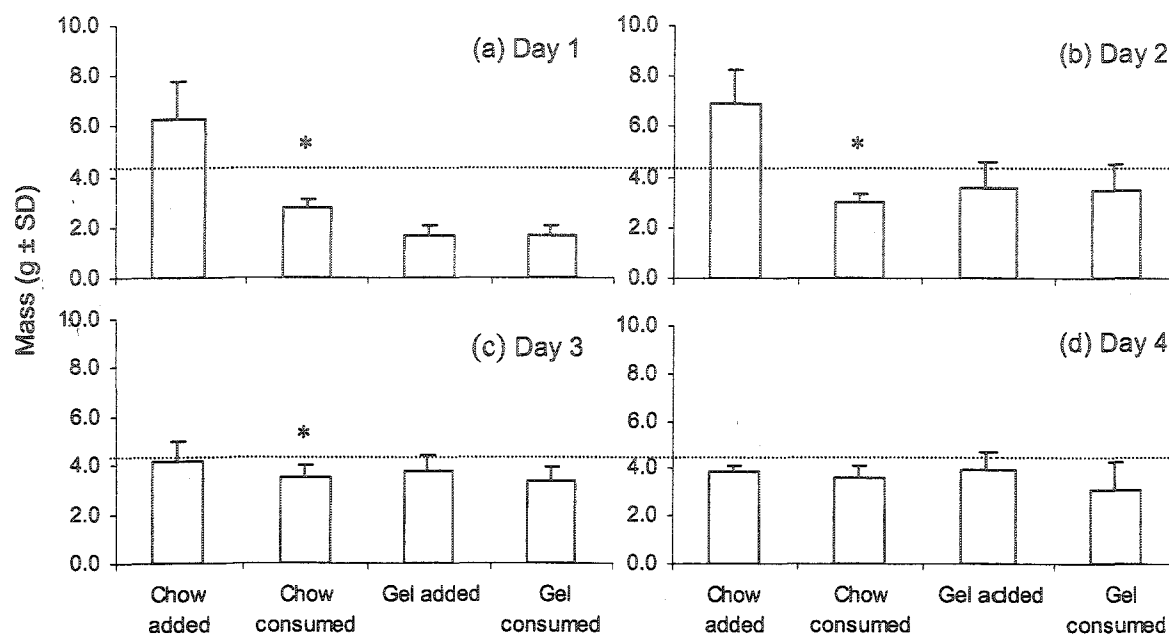


Fig 1. Mean dry mass ($\pm$ SD) of food provided and consumed by mice during four days of choice trials. (a) Day 1 – mice were provided with an excess of commercial dry chow and a small amount of 30% fish gel. (b) Day 2 – mice were provided with an excess of dry chow and a small amount of 35% fish gel. (c, d) Days 3 and 4 – mice were provided with similar amounts of dry chow and 35% fish gel. The dashed line represents the overall average mass of dry chow consumed by mice in the control group; asterisks indicate a significant difference in consumption of dry chow between mice in the fish diet treatment compared to those in the control group on each day determined using a t-test.

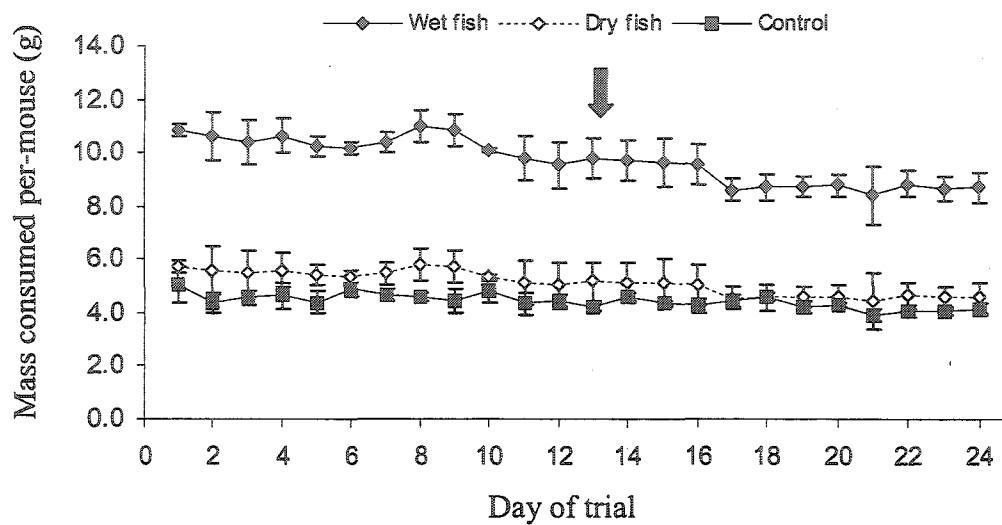


Fig 2. Average mass ($\pm$ SD) of food consumed per-mouse in groups fed commercial dry chow, or a gel diet containing 35% whole homogenized fish initially, followed by 50% fish beginning on day 13 (arrow). The dry mass of fish diet consumed was calculated based on a conversion factor of 53% moisture loss from the fish diet medium under laboratory conditions.

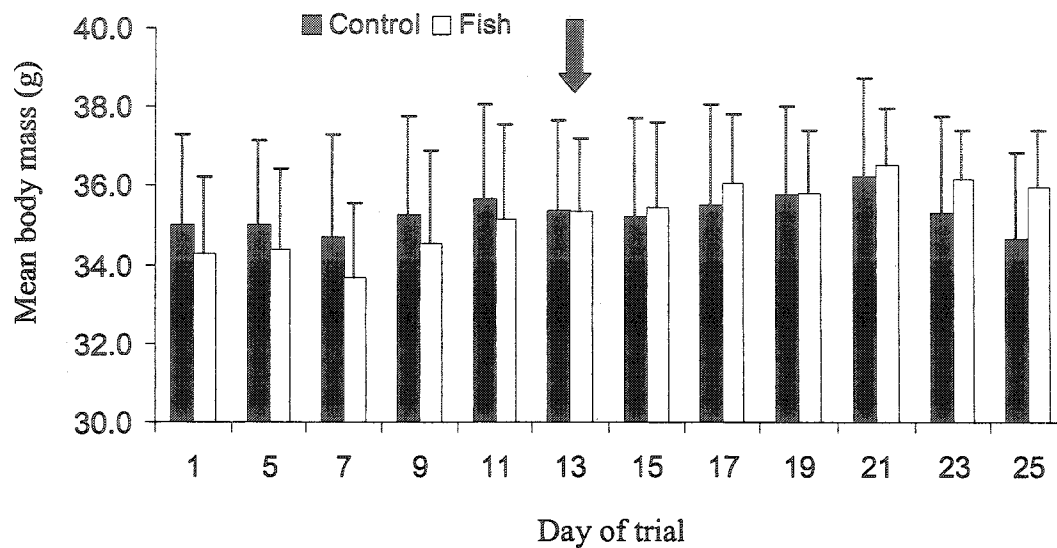


Fig 3. Body mass (+SD) of mice in groups fed commercial dry chow, or a gel diet medium containing 35% whole homogenized fish initially, followed by 50% fish beginning on day 13 of the trial (arrow). The measurement on day 25 is after one day of returning to *ad lib* dry chow consumption in both groups. There were 6 mice in the fish diet group and 5 in the control.

Table 3. Summary of two-way repeated measures ANOVA test for the effect of diet treatment and trial day on body mass of mice.

Source	Df ^a	F	P-value
Diet	1, 9	0.001	0.984
Trial day	7.7, 69	8.013	< 0.001
Diet x Trial day	7.7, 69	2.772	0.011

a – Huynh-Feldt correction for lack of sphericity used on degrees of freedom

CHAPTER 5

A diet of small-bodied fish and germline mutations at repetitive DNA loci in mice

Christopher M. Somers¹, Eduardo V. Valdes², Victoria A. Kjoss¹, Andre M.
Vallaincourt³, James S. Quinn¹

1 – McMaster University, Department of Biology, 1280 Main St. West, Hamilton, ON,
Canada, L8S 4K1

2 – Nutrition Centre, Metropolitan Toronto Zoo, 361A Old Finch Ave, Scarborough, ON,
Canada, M1B 5K7

3 – Ontario Ministry of Natural Resources, Sport Fish Monitoring Program, 125
Resources Rd., Etobicoke, ON, M9P 3V6

ABSTRACT

Herring gulls (*Larus argentatus*) nesting near industrial areas containing integrated steel mills were shown to have higher germline mutation rates at minisatellite DNA loci than those nesting in rural locations. Airborne or aquatic chemical contaminants were proposed as an explanation for these induced mutations, but the route of exposure was unknown. Follow-up experiments with sentinel lab mice determined that ambient air pollution in an industrial area could induce germline mutations, but an evaluation of genotoxicity associated with the diet of herring gulls has not previously been conducted. To address this issue, for 10 weeks we fed lab mice a diet containing 58% homogenised rainbow smelt (*Osmerus mordax*) and alewife (*Alosa pseudoharengus*) collected from Hamilton Harbour, a polluted industrial area on western Lake Ontario. We later bred the mice and screened pedigrees for mutations at expanded simple tandem repeat (ESTR) DNA loci. Mutation rates were compared to those in groups of mice fed a diet of 58% fish from Atlantic Canada, or commercial dry chow. Body mass and condition of adult mice did not differ among groups during the 10 weeks of diet treatment. Initial mass of pups in the groups fed fish-based diets from either Hamilton Harbour or the Atlantic Ocean was lower than in the control group. Levels of organo-chlorine contaminants were substantially higher in Hamilton Harbour fish than in those from the Atlantic Ocean used in the reference fish diet; however, germline mutation rates, although generally highest in the group fed Hamilton Harbour fish, were not significantly affected by diet treatment. Our findings suggest a possible contribution of dietary contaminants to the heritable

genetic effects previously observed in herring gulls, but their relative importance remains unclear.

INTRODUCTION

Herring gulls nesting in an industrial area containing two integrated steel mills in Hamilton Harbour, western Lake Ontario, were shown to have elevated germline mutation rates at minisatellite DNA loci compared to those nesting in rural areas on the Great Lakes or east coast of Canada (Yauk and Quinn 1996). Further screening of herring gull colonies at additional sites in both Canada and the United States revealed a general trend for mutation rates to be elevated in colonies located near steel mills, and demonstrated a proximity relationship, with the highest mutation rates detected in colonies that were closest to steel mill locations (Yauk et al. 2000). These studies suggested that chemical contaminant exposure associated with living and breeding in polluted industrial areas was causing the observed differences in germline mutation rates among gull colonies, and potentially affecting other nearby organisms, including humans. However, the relative importance of airborne or dietary routes of genotoxic contaminant exposure could not be determined.

In response to the findings from herring gull studies, we exposed sentinel lab mice *in situ* to ambient air in a polluted industrial area near two integrated steel mills, and simultaneously at a rural location removed from point sources of air pollution. This experimental approach allowed us to control food and water sources for the mice during environmental exposure, effectively isolating air quality as the only major variable that

differed between sites. Germline mutation screening at ESTR DNA loci (Kelly et al. 1989, Gibbs et al. 1993, Bois et al. 1998) in mouse pedigrees revealed a 1.5- to 2.0-fold elevation in mutation frequency in mice exposed at the industrial site compared to those at the rural site, providing evidence that industrial air pollution exposure can cause elevated heritable mutation rates (Somers et al. 2002). A subsequent sentinel mouse exposure at the same field sites confirmed that industrial air pollution can induce germline ESTR mutations, and further demonstrated that high-efficiency-particulate-air filtration reduced mutation induction at the industrial site but not at the rural site, implicating airborne particulate matter as an important contributor to the observed genetic effects (Somers et al. 2004). In concert, these studies provide very strong evidence that air pollution is an important route of genotoxic contaminant exposure.

Air pollution, however, is only one of two potentially major routes of chemical contaminant exposure for herring gulls or other organisms living in industrial areas. Aquatic environments in the Great Lakes can be contaminated with a range of chemical substances, and top predators, such as piscivorous birds and sport fish, are often highly exposed to these contaminants as a result of bioaccumulation in aquatic food webs (Ewins et al. 1994, Stewart et al. 2000). To our knowledge, the potential for germline mutations to be induced by exposure to chemical contaminants through consumption of forage fish species common in the diet of piscivorous birds in the Great Lakes has not been previously evaluated. Rainbow smelt (*Osmerus mordax*) and alewife (*Alosa pseudoharengus*) make up approximately 58% of the diet of herring gulls in Lake Ontario (Ryckman et al. 2000), suggesting that these species are of interest for genotoxicity testing. In this study, we examined the effects of a long-term diet containing 58% smelt and

alewife collected from Hamilton Harbour, a location with elevated mutation rates in local herring gulls, on germline ESTR mutation rates in mice.

METHODS

Fish collection and diet preparation - We used dip-nets and donations from anglers to collect 15 kg of rainbow smelt and 18 kg of alewife from Hamilton Harbour, a polluted industrial portion of western Lake Ontario (43°15'N, 70°51'W), between 04 April and 15 June 2001. Alewife were on average ($\pm$ SD) 15.6 ± 1.4 cm long, and weighed 28.0 ± 6.9 g; rainbow smelt were 13.1 ± 1.3 cm long and weighed 14.1 ± 5.1 g (based on 100 randomly selected individuals of each species). Atlantic rainbow smelt and herring (*Clupea harengus*) used to make a reference fish diet were obtained from commercial suppliers (Polar Brand International and High Seas Foods Ltd for smelt and herring, respectively). These species were selected because they are similar or identical to those collected from Hamilton Harbour, but were harvested from relatively uncontaminated marine environments. Atlantic herring were on average ($\pm$ SD) 27.8 ± 3.1 cm long and weighed 174.6 ± 60.5 g; Atlantic rainbow smelt were 15.2 ± 1.0 cm, and weighed 18.9 ± 3.7 g (based on 10 individuals of each species). Contaminant analysis of fish was conducted by the Ontario Ministry of Natural Resources (Sport Fish Monitoring Program). Each fish species was separately prepared into a gel diet medium for mice according to the procedures below.

Whole fish were ground using a Hobart 4346 mixer-grinder (Hobart Manufacturing, Troy, Ohio), followed by thorough homogenization with a Hobart VCM-

25 vertical cutter-mixer. Fish homogenate was combined with the other ingredients listed in Table 1, and mixed with the VCM-25 such that the final medium consisted of 58% whole fish by weight. Each diet medium was then poured into a large plastic container and set into a solid gel at 4°C. We made 20 kg of gel diet medium for each fish species, which was cut into 500g portions, sealed in plastic, and frozen at -20°C until the day before use. Nutritional analysis of fish diet gels was performed by the Agribrands Purina central laboratory, Strathroy, Ontario, Canada.

Mouse Feeding - We used outbred Swiss-Webster mice from Charles River Laboratories (St. Constance, Quebec, Canada). At age 7 to 9 weeks, mice were randomly divided into 3 treatment groups: (1) Control (11 males, 12 females) – fed Harlan Teklad 22/5 commercial rodent chow throughout the study, (2) Hamilton (20 of each sex) – fed the diet medium containing 58% smelt or alewife from Hamilton Harbour for 10 weeks, and (3) Atlantic (20 of each sex) – fed the diet medium containing 58% smelt or herring from Atlantic Canada for 10 weeks (see Table 1 for diet composition).

Mice were housed 3 or 4 same-sex individuals per cage, and all groups received pre-weighed quantities of food in 2 glass dishes placed on the floor at opposite ends of the cage. Tap water was provided *ad lib* from a sipper bottle. To adjust mice in the Hamilton and Atlantic groups to fish-based diets, we began by providing them with an excess of their normal commercial feed (Harlan Teklad 22/5 dry rodent chow), plus a small quantity of fish gel. For 7 days we incrementally reduced the amount of dry chow provided, and correspondingly increased the amount of fish gel. Day 1 of the feeding trial

started when we provided mice in the Hamilton and Atlantic groups with only fish-gel diet media.

For the 10-week (70-day) feeding trial we alternated the fish species provided within the Hamilton and Atlantic groups every three days. This resulted in mice in the Hamilton group receiving smelt and alewife for a total of approximately 35 days each, and the Atlantic group receiving smelt and herring for approximately 35 days each. Control mice received pre-weighed quantities of dry chow to mimic the handling and presentation of fish-gel in the other groups. Food was provided at 17:00 daily, and removed at 09:00 the following morning. We continuously increased the amount of food given over the 10 weeks to ensure that a small amount was left over after each feeding period. We monitored food consumption in cages receiving fish-based diets by pre-weighing the amount of gel medium provided, and then weighing the amount of food remaining in each cage the following morning. The observed mass of food remaining in cages is likely intermediate between dry mass and wet mass; however, the exact amount of moisture lost is uncertain. For consistency, to calculate daily consumption we converted both the wet mass of fish gel provided, and the mass of food remaining, to dry mass based on the moisture percentage determined for each gel type during nutritional analysis (see Table 2). This conversion is likely to cause a slight overestimate of food consumed by mice in both groups receiving fish-based diets. For the commercial chow control, we considered measurements of food provided and remaining to be dry mass.

At the end of the 10-week trial, all groups were returned to *ad lib* feeding on commercial dry chow (Harlan Teklad 22/5). We weighed mice weekly, and inspected them visually each day to monitor body condition.

Mouse breeding – 9 weeks after completing the feeding trial, males and females in each treatment group were randomly paired in breeding cages. This timing was selected to ensure that fertilizations resulted from mature sperm that would have been in diploid spermatogonial stem cells stages during diet treatment (Oakberg 1956). Breeding pairs were left together for 2 weeks, after which the males were euthanised and tail tissue collected for DNA extraction. We monitored females twice daily beginning on day 18 following pair formation, and recorded litter sizes and pup mass at 24, 48, 72, and 120 hours after birth. In a large number of cages females had at least partially cannibalized litters. Upon discovery of cannibalism, females and remaining pups from these litters were euthanised and tail / hind-end tissue sampled for DNA extraction. In the remainder of cages, females and all pups were euthanised when pups reached 5 days of age, and tail hind-end tissue was sampled for DNA extraction.

Genetic Analysis – Our approach to detecting germline ESTR mutations in mouse pedigrees has been described in detail elsewhere (Somers et al. 2002). In brief, DNA was extracted from tail tissue of parents and up to 6 pups (depending on litter size) in each family using a standard phenol/chloroform procedure. Six micrograms of DNA from each sample was digested to completion with HaeIII, size fractionated in 42cm, 0.8% agarose gels for 42 to 48 hours at 1.5 to 2.0 volts/cm, and then transferred to nylon membrane by Southern blotting. All lanes included 30ng of separately digested lambda phage DNA, added as an in-lane size standard for band migration distance (Galbraith et al. 1991). Membranes were hybridized with ³²P-labeled ESTR single locus probes Ms6-hm (Kelly

et al. 1989) and Hm-2 (Gibbs et al. 1993), ESTR multilocus probe MMS10 (Bois et al. 1998), and finally with lambda DNA. DNA profiles were visualized by autoradiography with high-resolution x-ray film (Kodak Biomax MR). Probe DNA was thoroughly stripped from membranes between hybridizations using 0.4N NaOH (42°C), followed by boiling 0.1% SDS.

Mutations were identified as bands in the DNA profiles of offspring that differed in size from their parental progenitors by 1.0mm or more as determined using the in-lane size standard (Somers et al. 2002, Yauk et al. 2002). We did not include mutant bands that appeared to be shared among littermates (clustered mutations), or extra non-allelic bands in the single locus profiles of offspring (somatic mutations). Mutation scoring was performed without knowledge of diet treatment, and a subset was verified by an independent observer.

Statistical Analysis – Body mass of mice was compared among treatment groups using repeated measures analysis of variance (RM-ANOVA). Individual mice were not uniquely marked, so we used the average mass from each cage as the repeated measure. This resulted in a subject sample size of 5 cages for each sex in the Atlantic and Hamilton diet groups, and 3 cages for each sex in the dry chow control group. We used a similar RM-ANOVA approach to compare food consumption on the last day of each week among groups using the average per-mouse consumption for each cage.

We compared the proportion of breeding pairs that produced at least one offspring (breeding success) among treatment groups using a chi-squared test. The number of pups per litter, and mass of pups within 24 hours of birth, were compared among groups using

one-way ANOVA tests. Scheffe post hoc testing was applied to any significant outcome from ANOVA.

Mutation rates were calculated as the number of mutant bands out of the total number of bands scored for all offspring in each treatment group (95% confidence intervals were estimated from the Poisson distribution). We compared mutation rates between groups using Fisher's Exact test. Application of Fisher's Exact test in this way resulted in a series of pair-wise comparisons, which we adjusted for using a sequential Bonferroni correction (Simes 1986). Power analysis on pair-wise comparisons of mutation frequencies was performed using a Z-test for two proportions. We also analyzed paternal and maternal per-band mutation rates for Ms6-hm and Hm-2 (combined) at the family level using two separate one-way ANOVA tests. This approach incorporates potential variance in mutation rates introduced by the exposure of different parents. To meet the assumptions of ANOVA, per-family mutation rates were arcsine transformed using an improved angular transformation procedure (Sokal and Rohlf 1981).

RESULTS

Body Mass – For most of the 10-week feeding trial and after the return to *ad lib* dry chow, male mice in the Atlantic group tended to be slightly heavier (3.6 to 7.9%) than those in the other two treatment groups (Fig 1A). Trial day explained a significant proportion of variance in male body mass (RM-ANOVA, $F_{14,140} = 378.7$, $P < 0.001$), but diet treatment did not (RM-ANOVA, $F_{2,10} = 3.36$, $P = 0.076$). Male mice increased in body mass by 33.3, 32.4, and 38.4% over the course of the 10-week feeding trial in the

control, Hamilton, and Atlantic groups, respectively. Similarly, measurement day explained a significant proportion of variance in female body mass (RM-ANOVA, $F_{14, 140} = 713.4$, $P < 0.001$), but diet treatment did not (RM-ANOVA, $F_{2, 10} = 1.09$, $P = 0.370$). Females increased in mass by 38.8, 35.7, and 38.6% over the 10-week feeding trial in the control, Hamilton, and Atlantic groups, respectively.

Food Consumption – Daily mass of food consumed differed by diet treatment (Fig 2A,B; RM-ANOVA, $F_{2, 20} = 97.9$, $P < 0.001$). Post hoc testing revealed that mice in the control group ate more on a daily basis than either fish-diet group, and that mice in the Hamilton group ate more than those in the Atlantic group. Males and females differed overall in the amount of food they consumed daily (RM-ANOVA, $F_{1, 20} = 3.81$, $P = 0.013$), with males consuming larger quantities of food. There was no significant interaction between diet treatment and sex (RM-ANOVA, $F_{2, 20} = 1.49$, $P = 0.079$, interaction term), indicating that males consumed more than females regardless of diet type (Fig. 2A,B).

Nutritional Analysis – basic analysis of nutritional parameters in the four types of fish gels is summarized in Table 2. A similar summary for the control dry chow can be found on the Harlan website at www.teklad.com/standard/index.htm (product 8640). The most notable difference between gels made from the different fish species was the total fat content, which was 8 to 51-fold higher in the Atlantic herring gel than those made from Great Lakes species, and over 3-fold higher than in the dry chow control. The diets were not isocaloric, with the Atlantic gels having a higher mass specific energy content than

either the Great Lakes gels or the dry chow control. The largest difference was generated again by the Atlantic herring diet medium.

Contaminant Analysis – Alewife and smelt from Hamilton Harbour had higher body burdens of all contaminants monitored than did herring and smelt from Atlantic Canada (Fig. 3). Alewife carried the highest body burdens measured; for example, total PCB levels were 11- to 23-fold higher in Hamilton Harbour alewife than in the Atlantic species. Mirex, photo-mirex, and toxaphene were present in all specimens collected from Hamilton Harbour, but were below the detection limits of 5, 4, and 50 ppb, respectively, in the Atlantic species.

Mouse breeding – 8/10 (80%) control, 17/20 (85%) Atlantic, and 11/18 (61%) Hamilton breeding pairs successfully produced litters. Although breeding success was lower in the group fed a fish-based diet from Hamilton Harbour, this difference was not significant ($X^2 = 3.06$, $df = 2$, $P > 0.05$). Average number of pups per litter was 6.0 ± 2.3 , 6.6 ± 2.7 , 7.6 ± 2.9 for the control, Atlantic, and Hamilton diet groups, respectively, and did not differ by diet treatment (ANOVA, $F_{2, 29} = 0.74$, $P = 0.486$). This analysis was likely confounded, however, by female cannibalism of pups, which did not permit precise litter size determination in some cases. Pup mass up to 24 hours post-delivery (control = 1.9 ± 0.2 , Hamilton = 1.6 ± 0.2 , Atlantic = 1.6 ± 0.2) varied by treatment group (ANOVA, $F_{2, 154} = 13.12$, $P < 0.001$); post-hoc testing revealed that pups delivered by females in the Hamilton and Atlantic groups were significantly smaller than those in the control group.

Germline Mutation Rates – Overall single locus mutation rates were 2.0-fold elevated in the Hamilton Harbour group over those in the dry chow control group, and 1.6-fold elevated over those in the Atlantic group. These elevations, however, were not significant following sequential Bonferroni correction (Table 3). Multilocus probe MMS10 did not reveal any clear patterns in mutation frequency among the three treatment groups (Table 3). Statistical power for detecting differences in single locus mutation rates between the Hamilton diet group and the dry chow control or the Atlantic diet groups was relatively low ($\beta = 0.384$ and 0.391 , respectively). To achieve $1-\beta = 0.70$ with $\alpha = 0.008$ based on the sequential Bonferroni correction applied here (Table 3), a sample size of approximately 250 offspring bands would be required in both the dry chow control and Hamilton diet groups. Assuming a constant magnitude of difference in mutation rates, this would require doubling the number of offspring/litters screened in the control group, in addition to increasing the Hamilton diet group by at least 2 more litters. For the comparison of Hamilton to Atlantic fish diet groups, approximately 440 offspring bands would be required in each group to achieve $1-\beta = 0.70$, with $\alpha = 0.01$ based on the sequential Bonferroni correction (Table 3). This would require ESTR mutation screening in twice as many litters in each treatment group as we assayed here.

High levels of polymorphism and heterozygosity at ESTR single loci Ms6-hm and Hm-2 allowed identification of the parental origin of all mutant bands. Paternal mutation rates in the Hamilton group were 2.3-fold elevated over the dry chow control, and 1.8-fold elevated over the Atlantic group; however, neither of these differences was significant following Bonferroni correction (Table 4). Statistical power to detect elevated paternal mutation rates in the Hamilton diet group was low, with $1-\beta = 0.396$

and 0.351 for comparisons with the control and Atlantic groups, respectively. To achieve $1-\beta = 0.70$ with $\alpha = 0.017$ (Bonferroni correction, Table 4) for comparing Hamilton and dry chow control diets would require scoring approximately 126 offspring bands in each group. In this case sample sizes would need to be increased by at least 3 more litters in the control group, and at least 1 additional litter in the Hamilton diet group. For $\alpha = 0.025$ in the comparison of Hamilton and Atlantic diets, approximately 204 offspring bands would need to be scored in each group. This would require screening twice as many offspring/litters in each group as we did here.

Maternal mutation rates followed a similar but less pronounced trend to that of the paternal rates, with those in the Hamilton diet group elevated 1.7-fold over the dry chow control, and 1.3-fold over the Atlantic group. None of the maternal differences among groups were significant before or after sequential Bonferroni correction (Table 4). Again, statistical detection power was quite low at $1-\beta = 0.125$ and 0.045 for the Hamilton group compared to the control and Atlantic groups, respectively. Sample size estimates based on these statistical power calculations indicated that 4.5- to 12.5-times as many litters/offspring as we screened would be required to achieve $1-\beta = 0.70$, assuming constant differences in mutation rates among treatment groups.

We also analyzed paternal and maternal per-band mutation rates at the family level using data for Ms6-hm and Hm-2 combined. This approach incorporates potential variance introduced by the exposure of different parents. Diet treatment did not explain a significant proportion of the variance in either paternal (ANOVA, $F_{2,32} = 1.15$, $P = 0.331$), or maternal mutation rates (ANOVA, $F_{2,31}$, $P = 0.267$). We pooled per-family mutation data in the dry chow and Atlantic diet groups and compared them to the

Hamilton Harbour group. This analysis also showed no significant difference in either paternal (t-test, $df = 31$, $P = 0.189$) or maternal per-family mutation rates (T-test, $df = 30$, $P = 0.119$).

Spontaneous lymphoma outbreak – Beginning 9 days after the feeding trial was completed, and ending after breeding, a total of 20 / 103 (19.4%) of the mice used in this study either died or had to be euthanised due to rapidly deteriorating body condition. Post-mortem inspection revealed splenomegaly, hepatomegaly, inflammation of one or both kidneys, and marked enlargement of numerous lymph nodes. All afflicted mice were diagnosed by a veterinary pathologist as having multicentric lymphoma. In all instances except one (18/20), the euthanized animal was the only affected individual in its cage. Disease incidence was not randomly distributed across the 3 diet treatment groups. Mice in the dry chow control group had the highest lymphoma frequency at 7/23 (30.4%), followed by the Hamilton fish-diet group at 11/40 (27.5%), and the Atlantic fish-diet group at 2/40 (5%; $\chi^2 = 7.16$, $df = 2$, $P < 0.05$; expected values generated based on equal incidence among groups and corrected for sample size).

In each of the dry chow control and Hamilton diet groups, three pairs of mice with at least one afflicted parent successfully produced offspring (the 2 Atlantic animals with the condition did not breed). We compared overall germline ESTR mutation rates between families with and without lymphoma within each diet treatment to determine whether disease status had any affect on germline ESTR mutation rates. In the dry chow control group, overall single locus mutation rates did not show an obvious trend, at 0.103 and 0.115 for normal and lymphoma-positive families, respectively (Fisher's Exact test, P

= 0.433). Mutation rates determined based on multilocus probe MMS10 were somewhat higher in the families of parents with lymphoma (0.008 normal, 0.015 with lymphoma), but this trend was not significant ($P = 0.514$). In the Hamilton fish-diet group, overall single locus mutation rates also showed no clear trend (normal = 0.213, lymphoma-positive = 0.259, $P = 0.246$). Similar to the dry chow control, mutation rates determined with multilocus probe MMS10 were somewhat elevated in the lymphoma-positive families (0.024) compared to the normal ones (0.016), but this trend was not significant ($P = 0.293$).

DISCUSSION

Zoo animals are commonly fed unusual diet items that have been homogenized into the gel format we used to deliver fish to mice in this study (Oyarzun and Santor 1980, Molitoris et al. 1998). This approach is well suited for rodent fish consumption studies because it provides flexibility in terms of the relative quantities of fish material that can be fed to experimental animals, while permitting supplementation for nutrition and palatability requirements. In our study daily consumption of each diet type differed among groups, as did some potentially important nutritional parameters, such as mass specific energy content and total fat levels. Despite these differences, body mass of male and female mice was not significantly affected by diet treatment. We initially interpreted this to mean that very high levels of fish (58% by wet mass) did not have any negative effects on the overall condition of adult mice in the Hamilton and Atlantic diet groups. Initial mass of pups in the Atlantic and Hamilton groups, however, was significantly

lower than in the dry chow control group, suggesting that adult females may have been affected by the high fish diet in some way that reduced the mass of their developing pups, but was not manifest as a change in adult body mass relative to the control group. Females returned to an *ad lib* diet of commercial dry chow for nine weeks prior to breeding, as well as during pregnancy, so there should have been ample opportunity to recover from any nutritional deficits incurred during the fish feeding trial. The smaller initial pup mass in the fish diet groups remains unexplained, but indicates that monitoring only body mass as a measure of condition in diet manipulation trials like this one may not be sufficient.

Overall single locus ESTR mutation rates in the group fed fish from Hamilton Harbour showed a trend to be elevated over both the Atlantic fish and dry chow control groups. The relative elevation of 1.6- to 2.0-fold was similar in magnitude to that detected in our previous sentinel mouse studies on urban/industrial air pollution exposure (Somers et al. 2002, 2004). Statistical analyses that accounted for multiple pair-wise comparisons among groups, however, showed that mutation rates in the Hamilton group were not significantly elevated. In addition, trends in mutation rates detected using multilocus ESTR probe MMS10 did not parallel those for ESTR single loci Ms6-hm and Hm-2, as they have in our previous studies (Somers et al. 2002, 2004). Statistical power analysis showed that it might be feasible with modest sample size increases to detect a difference in mutation rates between the Hamilton and dry chow control groups, but this was not the case for comparing Hamilton and Atlantic fish groups, which would require at least a doubling in the sample size.

Similarly, paternal mutation rates in mice fed fish from Hamilton Harbour showed a trend to be elevated over the Atlantic and dry chow control groups. Again, this 1.8- to 2.3-fold elevation was similar in relative magnitude to increases in paternal mutation rates detected in our previous studies of industrial air pollution (Somers et al. 2002, 2004), but was not significant following correction for several pair-wise comparisons among diet treatments. While only a modest increase in sample size would be required to demonstrate a significant difference in paternal mutation rates between the Hamilton and dry chow control groups, a doubling in sample size would be required for the comparison of the Hamilton and Atlantic groups. Perhaps more importantly, however, at the family level diet treatment did not explain significantly more of the variance in paternal mutation rates than the error term for variance within groups. This analysis is potentially more informative than that of the pooled mutation frequencies for all pups within each treatment group, because it incorporates variance in mutation rates introduced by the exposure of a number of different sires. This is an important consideration, particularly when using out-bred mice. The lack of significance for a paternal effect of diet therefore appears to stem from the combination of small sample sizes (low statistical power) and high variance in mutations rates. Consequently, we are not able to draw a firm conclusion regarding the effect of the diet of smelt and alewife from Hamilton Harbour on paternal ESTR mutation rates in mice. There was no statistical support of any kind for differences in maternal mutation rates among diet treatment groups.

PCBs, which are carcinogenic in lab animal tests (reviewed in Faroon et al. 2001), and the cause of many sport fish consumption advisories (Kassa and Bisesi 2001), were the major contaminants detected in Hamilton Harbour smelt and alewife. Average PCB

levels in alewife (454 ± 146 ppb) were high enough to border on the level of preliminary dietary restriction for humans in Ontario (500ppb; Health Canada). Despite this level of PCB contamination, the increase in germline mutations we detected in the group of mice fed smelt and alewife from Hamilton Harbour was not significant. Similar results were obtained in a field study of germline minisatellite mutations in barn swallows chronically exposed to high dietary PCB levels (Stapleton et al. 2001). In contrast, Hedenskog et al. (1997) detected significantly elevated mutation rates at ESTR single locus Pc-1 (Ms6-hm) in mouse pedigrees generated eighteen days after treatment of male mice with two intraperitoneal (IP) injections of 100mg/kg of a PCB mixture (Aroclor 1254). Each acute IP dose was 1250- to 5000-fold higher than the estimated daily dose of 0.02 to 0.08 mg/kg PCBs in our Hamilton fish-diet group (based on fish contaminant analyses and daily food consumption). In addition, we scheduled matings such that male mice were exposed during diploid spermatogonial stem cell stages of germ cell development, as opposed to meiotic and post-meiotic cell stages eighteen days post-treatment. It is therefore difficult to compare the outcome of these two mouse experiments. Given the widespread contamination of aquatic environments with PCBs, and the inconclusive nature of germline mutation screening in this study, we suggest that future research investigate the stage specificity and dose response of ESTR mutation induction in the male germline to PCB exposure under controlled conditions, similar to those described for ionizing radiation (Dubrova et al. 1998) and some chemical mutagens (Villerino-Guell et al. 2003). This is also the case for other potentially carcinogenic chemicals detected in the fish collected from Hamilton Harbour (e.g., mirex, toxaphene, and DDE).

In our study the element of control provided by a sentinel animal approach (Reichrtova et al. 1995, Reymao et al. 1997, Somers et al. 2002) was partially compromised by a lymphoma outbreak that affected nearly 20% of the animals. The origin of this condition is unknown, but other researchers using this supplier and strain of mice have recently reported similar outbreaks that were likely caused by endogenous murine retroviruses (Taddesse-Heath et al. 2000, Ceccarelli and Rozengurt 2002). Murine retroviruses can behave as mutagens through insertion of virus-encoding DNA into the host genome (Hansen et al. 2000). Oncogenesis is the result of such insertions that disrupt loci associated with normal cell cycle regulation and gene expression (Weidhass et al. 2000, Jin et al. 2003). Intuitively, retroviral insertions in mouse germ cells might have the potential to affect ESTR mutation rates. Comparisons of mutation rates in normal and lymphoma-positive animals within the dry chow control and Hamilton fish-diet groups did not reveal any significant effect of the disease. In addition, there was no relationship between germline mutation rates and lymphoma incidence. For example, lymphoma was most prevalent in the dry chow control group, but all measures of germline ESTR mutation rates were lowest in these mice. Similarly, lymphoma frequency was the lowest in the Atlantic fish-diet group, but germline mutation rates were intermediate between the Hamilton and dry chow control groups. Other than causing mortality, and thus a reduction in sample size, we are unable to identify any specific effects of the lymphoma outbreak on the interpretation of our results.

Consumption of sport fish from the North American Great Lakes, and some fish species raised in aquaculture facilities, has been identified as a potentially major source of chemical contaminant exposure for humans (Kearney *et al.* 1999, Schantz *et al.* 1999,

Nadon *et al.* 2002, Cole *et al.* 2002, Hites *et al.* 2004). Correspondingly, all previous research using rodents to evaluate biological responses to contaminated fish diets has focused on large-bodied, predatory fish species (e.g., Cleland *et al.* 1987, 1988, Beattie *et al.* 1996, Arnold *et al.* 1998, Gerstenberger *et al.* 2000). The general conclusion from studies of Great Lakes salmon fed to rats is that toxicological risk to humans is minimal provided that consumption advisories are followed (Feeley *et al.* 1998). This is not the case, however, for piscivorous wildlife species, including a number of sport fish and waterbirds, which do not have the option to modify their diets based on pollution levels. The high body-burden of contaminants commonly measured in birds and sport fish in the Great Lakes is likely the direct result of a diet high in small-bodied fish species. Our study is the first to apply a rodent feeding trial to test a hypothesis regarding toxicological effects from a diet comprised of fish species that are important to wildlife. Despite the high dietary levels of fish used, and levels of chemical contaminants that approached those observed to produce reproductive effects in other mammals (e.g., PCBs in mink; Restum *et al.* 1998), we did not detect any statistically significant effects of diet on germline ESTR mutation rates. However, given the low statistical power in our comparisons, and the fact that paternal mutation rates in mice fed fish from Hamilton Harbour were 1.8- to 2.3-times as high as those in the other groups, we are unable to exclude diet as a factor contributing to germline mutations in polluted areas.

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Table 1. Composition of fish-based diets fed to adult mice in the Hamilton Harbour and Atlantic treatment groups for 10 weeks. In addition to the ingredients listed below, Fish diet media were also supplemented with 0.12 IU / g vitamin E.

Ingredient	Quantity (g / kg)
^a Fish homogenate	580
^b Ground chick chow	125
^c Ground mouse chow	125
^d Toronto Zoo carnivore supplement	20
Mono dical phosphate	10
Thiamin	0.3
Gelatin	60
Distilled water (100°C)	80

a - Mechanically homogenized whole rainbow smelt or alewife, and rainbow smelt or herring for the Hamilton Harbour and Atlantic diet groups, respectively.

b - Purina Mills chick chow SG5065

c - Purina Mills mouse chow 9F5020

d - Roche Ltd

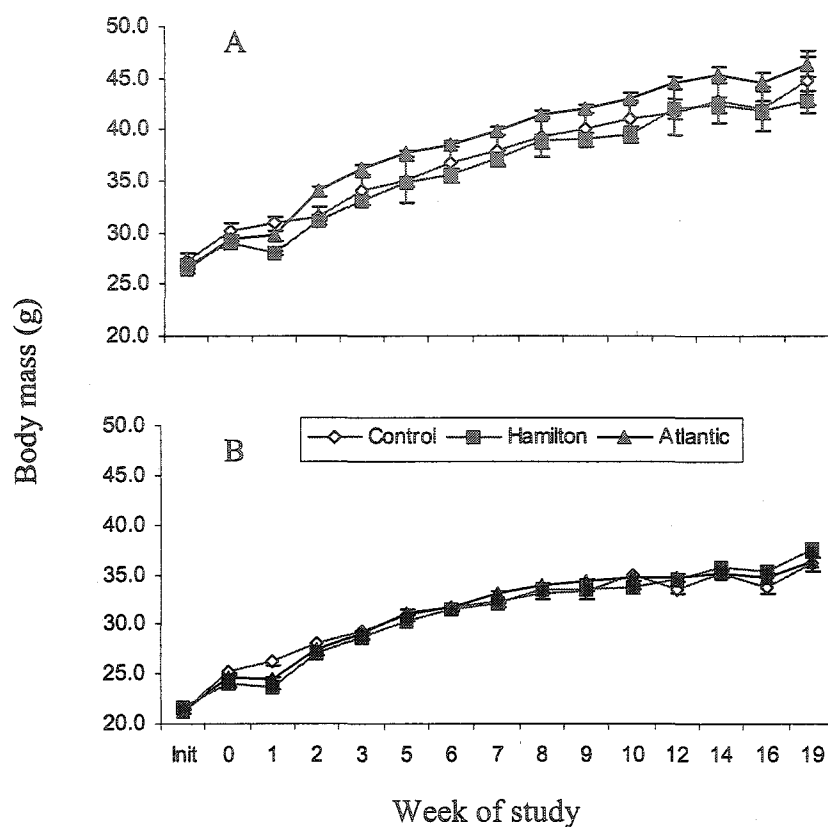


Fig 1. Average ($\pm$ SE) body mass of male (A) and female (B) mice on three different diets over the course of the study. Init = average mass before beginning experimental diet treatments. Week 0 is following habituation to fish diets; weeks 1 through 10 represent the feeding trial portion of the experiment, and weeks 12 through 19 the period after returning to *ad lib* commercial dry chow in all groups prior to breeding. The values presented here are based on averages for each cage, which were the units of repeated measure in statistical analyses.

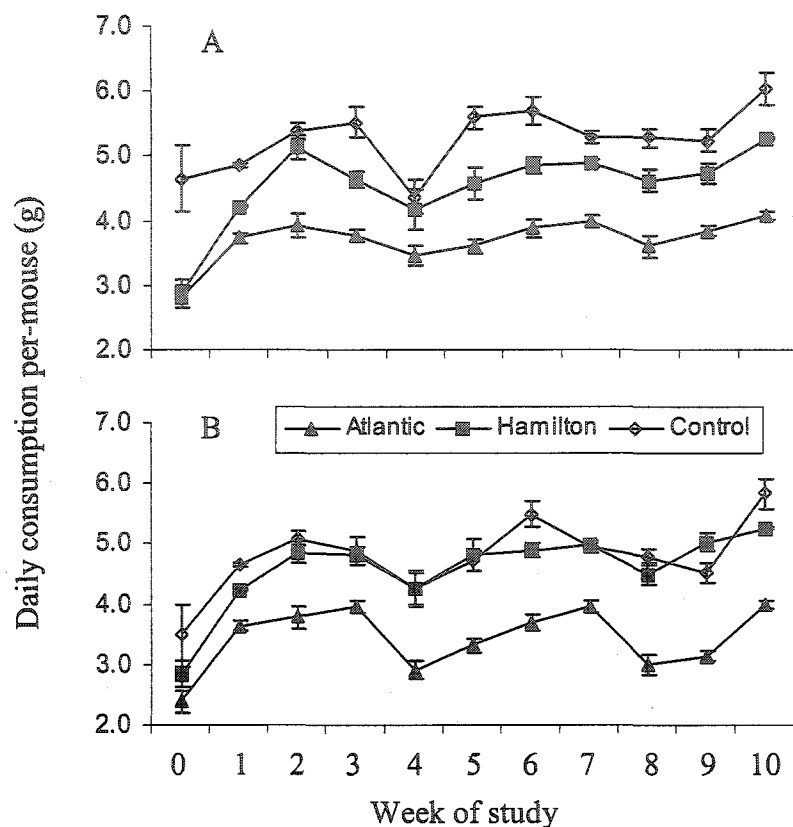


Fig 2. Mean ($\pm$ SE) dry mass of food consumed per-mouse for male (A) and female (B) mice in groups fed three different diet types. The values presented here are based on averages for each cage, which were the units of repeated measure in statistical analyses. Week 0 is consumption on the first day that mice in the Hamilton and Atlantic groups were fed only fish gel diet media following a 7-day habituation period as described in the methods section. Weeks 1 through 10 represent consumption values measured on the last day of each week of the feeding trial.

Table 2. Nutritional parameters (dry matter basis) of fish diet media used to feed adult mice in the Hamilton Harbour and Atlantic treatment groups.

Parameter	Units	Hamilton Harbour		Atlantic	
		Smelt	Alewife	Smelt	Herring
Energy	Kcal / g	3.82	3.32	4.52	5.81
*Moisture	g / kg	459	476	508	527
Crude protein	g / kg	420	439	425	457
Fat	g / kg	3	19	6	154
Crude fiber	g / kg	15	23	10	38
Acid detergent fiber	g / kg	83	36	20	30
Sodium	g / kg	3.0	3.2	3.3	3.6
Calcium	g / kg	13.5	18.3	14.8	14.4
Magnesium	g / kg	1.1	1.3	1.2	1.5
Potassium	g / kg	7.2	8.2	7.9	8.7
Phosphorous	g / kg	12.0	14.3	13.6	14.0
Copper	mg / kg	13	15	16	17
Iron	mg / kg	349	412	392	444
Zinc	mg / kg	144.2	171.8	156.5	164.9
Manganese	mg / kg	90.6	97.3	95.5	99.4
Vitamin A	IU / g	66.4	57.2	69.4	56.3
Vitamin C	mg / kg	< 4.0	< 4.0	< 4.0	< 4.0
Vitamin D	IU / g	5.1	7.2	6.4	10.3
Vitamin E	mg / kg	458	382	443	374

*moisture content is given for diet media as presented to mice for feeding.

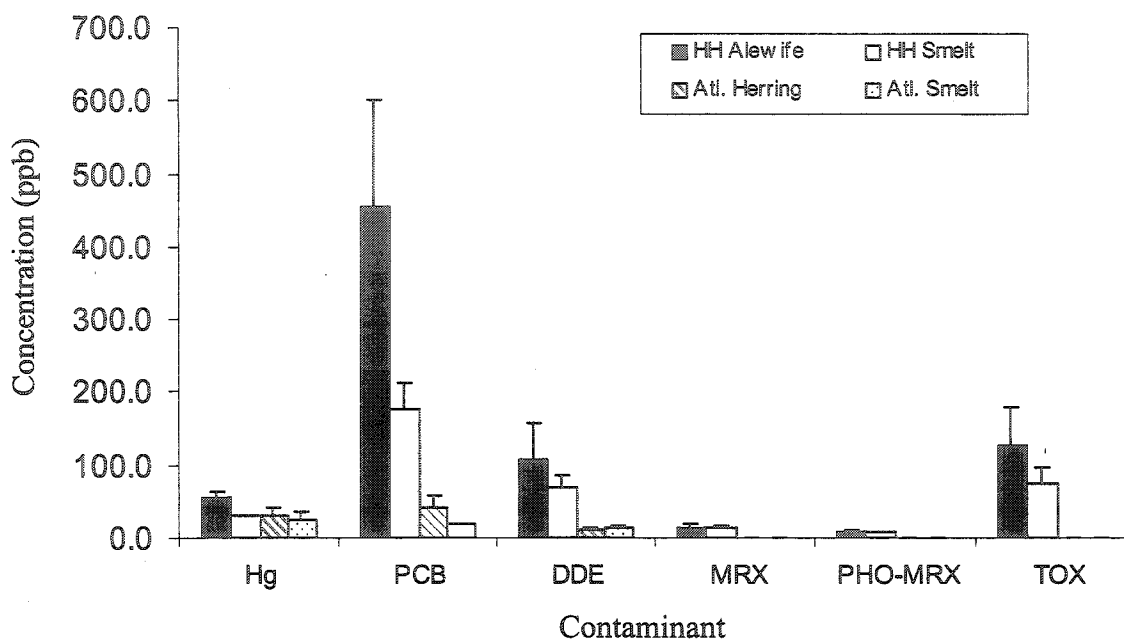


Fig 3. Average (+SD) whole-body burden of six contaminants in the four fish species fed to mice. HH alewife and smelt were collected from the Hamilton Harbour area of western Lake Ontario, whereas Atl. species were purchased from commercial suppliers fishing in the Newfoundland and Prince Edward Island areas of Atlantic Canada. These substances were selected for display from a general screening of 19 contaminants routinely monitored in Great Lakes sport fish because of health concerns associated with human fish consumption. Hg = mercury, PCB = total polychlorinated biphenyls, DDE = dichlorodiphenyldichloroethylene, MRX = Mirex, PHO-MRX = photo-Mirex, TOX = toxaphene.

Table 3. Overall germline ESTR mutation rates in 3 groups of mice fed different diets.

Group	Probe	Litters	Pups Scored ^a	Bands Scored ^{a,b}	Mutant Bands	Mutation Rate (95% CI) ^c	Comparison ^d	P-Value ^e	Corrected P-Value ^f
1) Control	Ms6-hm	7	36	73	9	0.123 (0.056, 0.235)			
	Hm-2	7	33	56	5	0.089 (0.028, 0.210)			
	Single locus			129	14	0.109 (0.059, 0.183)	1 : 2	0.103	0.309
	MMS10	7	36	528	6	0.011 (0.004, 0.025)	1 : 2	0.112	0.224
2) Atlantic	Ms6-hm	15	73	148	21	0.142 (0.088, 0.217)			
	Hm-2	15	57	88	11	0.125 (0.062, 0.224)			
	Single locus			236	32	0.136 (0.093, 0.192)	2 : 3	0.023	0.115
	MMS10	15	73	1367	20	0.015 (0.009, 0.029)	2 : 3	0.115	0.114
3) Hamilton	Ms6-hm	11	51	124	25	0.202 (0.130, 0.298)			
	Hm-2	11	43	69	17	0.246 (0.143, 0.395)			
	Single locus			193	42	0.218 (0.156, 0.294)	1 : 3	0.014	0.084
	MMS10	11	51	891	16	0.018 (0.012, 0.029)	1 : 3	0.076	0.304

a – In some pedigrees 1 or 2 alleles at ESTR single loci Ms6-hm and Hm-2 were not detectable in all adults, resulting in a variable number of pups and bands scored, particularly for Hm-2. In addition, Ms6-hm periodically detected more than 2 bands in the parents and pups of some pedigrees. These extra bands were included in mutation scoring when somatic mosaicism in pups was eliminated as an explanation.

b – Ms6-hm detected bands from a second, independent locus in some pedigrees resulting in more than 2 alleles scored per pup using this probe in some cases.

c – 95% confidence intervals estimated from the Poisson distribution.

d – Groups compared corresponding to numbers listed in the first column.

e – Probability value for one-tailed Fisher's Exact test.

f – Probability value for Fisher's Exact test after Sequential Bonferroni correction for 6 pair-wise comparisons (3 treatment groups x 2 probe types).

Table 4. Paternal and maternal germline ESTR mutation rates in mice fed 3 different diets. Footnotes are as in Table 3.

Group	Bands ^{a,b} Scored	Mutant Bands	Mutation Rate (95% CI) ^c	Comparison ^d	P-Value ^e	Corrected P-Value ^f
PATERNAL						
1) Control	72	8	0.111 (0.047, 0.220)	1 : 2	0.153	0.153
2) Atlantic	112	16	0.143 (0.081, 0.233)	2 : 3	0.039	0.078
3) Hamilton	107	27	0.252 (0.166, 0.367)	1 : 3	0.030	0.090
MATERNAL						
4) Control	57	6	0.105 (0.038, 0.231)	4 : 5	0.165	0.165
5) Atlantic	124	16	0.129 (0.074, 0.210)	5 : 6	0.122	0.244
6) Hamilton	86	15	0.174 (0.097, 0.288)	4 : 6	0.107	0.321

f – Sequential Bonferroni-corrected probability value for Fisher's Exact test in the 3 pair-wise comparisons of paternal and maternal mutation rates indicated.

CHAPTER 6

GAMMA-RADIATION INDUCED HERITABLE MUTATIONS AT REPETITIVE
DNA LOCI IN OUT-BRED MICE

C. M. Somers¹, R. Sharma², J. S. Quinn³, and D. R. Boreham⁴

^{1,2,3}McMaster University, Department of Biology, 1280 Main St. West, Hamilton,
Ontario, Canada L8S 4K1

⁴ McMaster University, Medical Physics and Applied Radiation Sciences Unit, 1280
Main St. West, Hamilton, Ontario, Canada L8S 4K1

Running Head: gamma-radiation and heritable DNA mutations in mice

¹Corresponding Author: Christopher M. Somers, McMaster University, Department of
Biology, 1280 Main St. West, Hamilton, Ontario, Canada, L8S 4K1

Phone: (905) 525 – 9140 x 24032

Fax: (905) 522 – 6066

e-mail: somerscm@mcmaster.ca

ABSTRACT

Recent studies have shown that expanded-simple-tandem-repeat (ESTR) DNA loci are efficient genetic markers for detecting radiation-induced germline mutations in mice. Dose responses following irradiation, however, have only been characterized in a small number of inbred mouse strains, and no studies have applied ESTRs to examine potential modifiers of radiation risk, such as adaptive response. We gamma-irradiated groups of male out-bred Swiss-Webster mice with single acute doses of 0.5 and 1.0 Gy, and compared germline mutation rates at ESTR loci to a sham-irradiated control. To test for evidence of adaptive response we treated a third group with a total dose of 1.1 Gy that was fractionated into a 0.1 Gy adapting dose, followed by a challenge dose of 1.0 Gy 24 hours later. Paternal mutation rates were significantly elevated above the control in the 0.5 Gy (2.8-fold) and 1.0 Gy (3.0-fold) groups, but were similar to each other despite the difference in radiation dose. The doubling dose for paternal mutation induction was 0.26 Gy (95% CI = 0.14 to 0.51 Gy). Males adapted with a 0.1 Gy dose prior to a 1.0 Gy challenge dose had mutation rates that were not significantly elevated above the control, and were 43% reduced compared to those receiving single doses. We conclude that pre-meiotic male germ cells in out-bred Swiss-Webster mice are sensitive to ESTR mutations induced by acute doses of ionizing radiation, but mutation induction may become saturated at a lower dose than in some strains of inbred mice. Reduced mutation rates in the adapted group provide intriguing evidence for suppression of ESTR mutations in the male germline through adaptive response. Repetitive DNA markers may be useful tools

for exploration of biological factors affecting the probability of heritable mutations caused by low-dose ionizing radiation exposure. The biological significance of ESTR mutations in terms of radiation risk assessment, however, is still undetermined.

1. INTRODUCTION

Mouse expanded simple tandem repeat (ESTR) DNA loci are long tandem arrays of 4- to 6-base pair repeat units that are unstable in the germline, and mutate primarily by insertion or deletion of a number of repeat units. Mutations of this kind can be detected in the progeny of experimental animals by pedigree DNA profiling using single and multilocus DNA fingerprinting [1 - 3]. Clear dose-response relationships for ESTR mutation induction have been demonstrated in pre-meiotic male germ cells following acute and chronic ionizing radiation exposure [4, 5], and chronic neutron irradiation [5; but see also 6 - 8]. The doubling dose for ESTR mutation induction in CBA/H mice following acute gamma-irradiation was determined to be 0.33 Gy, similar to estimates for coding regions of the mouse genome obtained using phenotypic markers in the specific locus test [4]. The major advantage of the ESTR assay in all of these studies was that it required only modest numbers of experimental animals, and detected statistically significant increases in mutation frequency at low radiation doses of 1 Gy or less. This sensitivity makes ESTRs much more efficient genetic markers for studying heritable mutation processes than any previous *in vivo* rodent system [4, 5, 9, reviewed in 10].

Biological factors that influence ESTR mutation frequency in the mouse germline have not been well investigated. ESTR mutation rates following low-dose irradiation have only been quantified in a small number of highly inbred mouse strains [4, 5, 9], or F1 hybrids [11, 12], and both spontaneous and radiation-induced mutation frequencies

varied by strain [9]. This suggests that genetic background influences tandem-repeat instability in the mouse germline, and identifies the need to characterize responses to low-dose irradiation at ESTR loci in a wider variety of mice, including more genetically variable out-bred strains. In addition, ESTRs may provide a valuable tool for investigating biological processes that modify low-dose radiation risk *in vivo*, such as genetic susceptibility, and adaptive response, but to date they have not been applied in this context.

Adaptive response, where low doses of radiation induce resistance to further genetic damage caused by subsequent higher doses, has been reported in a number of mammalian cell types *in vitro* using a variety of measures of DNA damage and genomic instability [reviewed in [13 - 15]. The adaptive response has also been characterized in single-celled organisms such as bacteria [16] and yeast [17 - 20]. These studies at the cellular level suggest that adaptive response may reduce the risk associated with whole-body, low-dose radiation exposure in humans and other biota, most likely through activation of efficient DNA repair systems [21, 22]. By far the majority of research to date, however, has focused on *in vitro* model systems, and much less is known about adaptive response function *in vivo*.

A small number of studies have demonstrated adaptive response at the whole-animal level in vertebrates. Some of the documented positive effects of pre-irradiation have included: reduced short-term mortality rates in mice [23 - 26], reduced frequency of chromosome abnormalities in mice [27, 28] and humans [29 - 31], and reduced risk of cancer development in mice [32 - 34]. These studies provide clear evidence for reduction and/or delay of genetic instability and ultimately disease through adaptive response;

however, the majority of both *in vivo* and *in vitro* studies have focused on somatic markers of DNA damage or cancer. An area of research that has received much less attention is the modification of risk associated with radiation-induced heritable mutations through adaptive response in mammalian germ cells.

Cai and Liu [35] showed that male mice whole-body irradiated with an adapting dose of 0.05 Gy, followed by a challenge dose of 0.75 Gy 3 hours later, had significantly reduced rates of spermatocyte chromatid aberrations compared to males given the challenge dose alone. Similarly, male mice adapted with doses ranging from 0.01 to 0.15 Gy, followed by a challenge dose of 1.5 Gy 3 hours later, showed significant reductions in spermatocyte chromatid aberrations and spermatogonial translocations compared to those given only the challenge dose. Dominant lethal mutations were also reduced in adapted compared to non-adapted males when they were mated to non-irradiated females such that fertilizing sperm were irradiated during pre-meiotic stages of development [36, 37]. These studies provide evidence for adaptive response in pre-meiotic male germ cells; however, to date it has not been determined whether this translates into a reduced rate of transmissible mutations inherited by their offspring.

Based on the issues outlined above, our study had two major objectives: first, to examine the dose-response to ionizing radiation at ESTR loci in pre-meiotic germ cells in male mice of an out-bred strain, and second, to test for evidence of ESTR mutation reduction in the paternal germline through adaptive response.

2. MATERIALS AND METHODS

2.1. Mouse irradiation and breeding - The mice used in this study were an out-bred Swiss-Webster strain from an exogenous pathogen-free colony (Taconic Breeding

Laboratories, Germantown, NY, USA). We had four treatment groups of 10 males (7 to 9 weeks old, 33 to 40 g) each: (i) 0 Gy (sham-irradiated control), (ii) 0.5 Gy, (iii) 1.0 Gy, and (iv) 0.1 + 1.0 Gy. Irradiated animals were given acute doses of whole-body radiation from a broad-beam cesium-137 source, and those in the control group were sham-irradiated by being placed in the Cs-137 unit but the source was not exposed. Mice in the 0.5 and 1.0 Gy groups received one acute dose, whereas those in the 0.1 + 1.0 Gy group received an adapting dose of 0.1 Gy, followed by a larger challenge dose of 1.0 Gy 24 hours later. All radiation doses were delivered at 0.36 Gy per minute. Dosimetry was performed using a pre-calibrated Farmer dosimeter with a 0.6cc chamber. The chamber was calibrated at the National Research Council of Canada Laboratory.

Irradiated males were bred to untreated females 9 weeks post-irradiation. This delay in breeding was selected to ensure that fertilizations resulted from mature sperm that developed from irradiated 2n-spermatogonial stem cells [4]. Males were euthanized 14 days post-pairing, and cardiac tissue collected for DNA extraction. Numbers of pups per litter and the mass of pups at 0, 24, 48, and 96 hours after birth was measured in each group. We euthanized the females and all pups at 96 hours following delivery, and sampled cardiac tissue from females and tail / hind-end tissue from pups for DNA extraction. All animal procedures were approved by the McMaster University Animal Research Ethics Board following the guidelines of the Canadian Council on Animal Care.

2.2. Genetic analyses – The approach we used to detect germline mutations at ESTR loci has been described elsewhere [38]. In brief, genomic DNA was phenol/chloroform extracted from tissue samples of adults and 3 to 6 pups (based on litter size) from mouse

families in each treatment group, digested to completion (6 ug) with Hae III, and size-fractionated in 42-cm, 0.8 % agarose gels for 42 to 50 hours (1.5 to 2 volts/cm). DNA was then transferred to nylon membrane by Southern blotting (Hybond-XL, Amersham-Pharmacia), and DNA profiles produced by hybridization with ^{32}P -labelled synthetic single-locus ESTR probes *Ms6-hm* [1] and *Hm-2* [2], and multilocus probe MMS10 [3]. DNA profiles were visualized using autoradiography. In the majority of families, some *Ms6-hm* alleles were too small (range 1 to 3.5 kb) for detection using this electrophoresis protocol. To try and detect these smaller *Ms6-hm* fragments we generated an additional DNA profile for each family by running 25-cm, 1.0 % agarose gels for 20 to 24 hours at 1.0 to 1.5 volts/cm, which were then Southern blotted and probed with *Ms6-hm*. All samples were run with 30 ng of digested lambda phage DNA as an in-lane size standard [39].

We identified mutations as bands in offspring that deviated by 1.0 mm or more relative to their parental progenitors based on comparison to the in-lane size standard [12, 38]. Any bands that were detected by both single and multilocus ESTR probes were included in the single locus mutation rate only. Mutation events resulting in identical mutant bands shared among littermates, or extra, non-allelic bands in single locus profiles (somatic mutations during embryogenesis), were not included in our analyses. Mutation scoring was performed without knowledge of treatment group, and verified by an independent observer.

2.3. Statistical Analyses - Observed litter sizes and mean mass of pups in each litter were compared among treatment groups using a one-way analysis of variance (ANOVA). Mutation rates for each treatment group were calculated as the number of mutant bands

out of the total scored (95% confidence intervals based on the Poisson distribution), and compared using two-tailed Fisher's Exact Probability Tests [40]. Use of Fisher's Exact Test in this way resulted in multiple pair-wise comparisons (each treatment group to the control), so we present both the calculated two-tailed P-value, and the sequential Bonferroni-corrected P-value [41] for each Fisher's Exact Test. In addition, we also analyzed paternal and maternal per-band mutation rates at the family level using two separate one-way ANOVA tests. For the majority of litters, per-family mutation rates were calculated based on data for ESTR single locus probes *Ms6-hm* and *Hm-2* combined. In some families, however, only one ESTR probe provided useful mutation data. We included these families in ANOVA following confirmation that their mutation rates did not fall outside of the range calculated for families scored at both ESTR single loci. To meet the assumptions of ANOVA, per-band mutation rates for all families were arcsine-transformed using an improved angular transformation procedure [40]. We used general contrast statements to test specific hypotheses regarding differences among treatment groups.

The doubling dose was estimated for ESTR loci *Ms6-hm* and *Hm-2* based on the per-Gy rate of paternal mutation induction relative to the control in 0.5 and 1.0 Gy treatment groups, assuming a linear relationship between radiation dose and mutation rate as previously reported [4]. We present the mean doubling dose value and overall 95% confidence intervals.

3. RESULTS

Body mass and condition did not differ among the four groups of irradiated adult males up to 21 days post treatment (data not shown). Observed litter sizes were $12.6 \pm$

1.8, 13.7 ± 2.3 , 12.7 ± 2.1 , and 12.9 ± 2.3 for control, 0.5 Gy, 1.0 Gy, and 0.1 + 1.0 Gy groups, respectively, and did not differ by treatment (ANOVA, $F = 0.70$, $df = 3$, $P = 0.556$). The mean body mass of pups in litters in each treatment group was similar at all four intervals measured over the first 96 hours after birth (data not shown).

Single and multilocus ESTR probes detected an overall elevation in germline mutation frequency of more than two-fold in 0.5 and 1.0 Gy irradiated groups compared to the control (Fig. 1 a, b). The relative elevation above the control was similar in both of these groups despite the increase in radiation dose, and was statistically significant for both types of ESTR probe before, and at least one type of ESTR marker after sequential Bonferroni correction (Table 1). In contrast, a smaller, non-significant elevation in mutation frequency above the control was detected with single locus ESTR probes in the 0.1 + 1.0 Gy group, and no apparent elevation with multilocus probe MMS10 (Fig 1 a, b; statistical summaries in Table 1).

High levels of polymorphism at ESTR single loci *Ms6-hm* and *Hm-2* allowed us to determine the parental origin of mutant bands. Based on pooled data from all pups in each treatment group, paternal mutation frequency was significantly elevated above the control by approximately three-fold in 0.5 and 1.0 Gy irradiated groups (Fig. 2). Again, both of these groups were elevated to a similar level above the control despite the difference in radiation dose (statistical summaries in Table 2). The average doubling dose for paternal mutation induction at *Ms6-hm* and *Hm-2* was 0.26 Gy (95% CI = 0.14 to 0.51 Gy) based on mutation rates in the control, 0.5 Gy, and 1.0 Gy groups. The paternal mutation rate in the 0.1 + 1.0 Gy group was not significantly elevated above the control (Fig. 2), and was 42% and 45% decreased relative to the 0.5 or 1.0 Gy groups,

respectively (Fisher's Exact Test, $P = 0.080$, and $P = 0.089$, respectively). The paternal mutation rate in the 0.1 + 1.0 Gy group was significantly (43%) reduced relative to the 0.5 Gy and 1.0 Gy groups pooled (Fisher's Exact Test, $P = 0.048$). Maternal mutation rates did not differ among the four treatment groups (Fig. 2; statistical summaries in Table 2).

Analyses of paternal mutation rates on a per-family basis, which incorporates potential sire-to-sire variance, revealed a significant overall effect of radiation treatment (ANOVA, $F_{3, 39} = 4.31$, $P = 0.011$). Using general contrast statements, we compared the control group to all irradiated groups and found only a borderline difference in mutation rates (Scheffe's $F = 2.74$, $P = 0.057$). This was likely the result of lower mutation rates in the 0.1 + 1.0Gy adapted group than in the groups receiving single acute radiation doses. Paternal mutation rate in the 1.0Gy group did not differ significantly from that of the 0.1+1.0Gy adapted group (Scheffe's $F = 1.58$, $P = 0.210$); however, the control and 0.1 + 1.0Gy adapted groups together had lower paternal mutation rates than the 0.5Gy and 1.0Gy groups (Scheffe's $F = 4.54$, $P = 0.008$). Maternal per-family mutation rates did not vary among treatment groups (ANOVA, $F_{3, 38} = 0.44$, $P = 0.726$).

4. DISCUSSION

In our study germline ESTR mutation rates did not increase in a strictly dose-dependent manner. The similar elevation in mutation frequency above the control in both the 0.5 and 1.0 Gy groups was unexpected based on the linear dose response for ESTR mutations observed in inbred CBA/H mice using the same doses of ionizing radiation [4, 42]. One possible explanation for this difference is that the out-bred Swiss-Webster mice we used were slightly more radiosensitive than CBA/H mice, causing mutation induction

to become saturated at an unknown dose less than 0.5 Gy. Other studies of ESTR mutation induction have shown that radio-sensitivity varies by mouse strain [9], and that saturation in mutation rates can occur in response to both radiation [43] and chemical mutagen exposure [44]. Possible alternative explanations also include: (a) that the use of genetically variable mice and the reduction in sample sizes caused by ESTR alleles that were unsuitable for mutation scoring introduced greater variance in induced mutation rates in our study, obscuring a more linear dose response relationship; or (b) that inducible DNA repair processes and / or apoptosis pathways in our out-bred mouse line respond to radiation damage differently than in some inbred strains.

Despite the minor difference in the nature of the dose response characterized, the average doubling dose of 0.26 Gy for mutation induction in spermatogonial stem cells estimated from our data is similar to the estimate of 0.33 Gy previously obtained for ESTRs using inbred mice [4]. The close agreement between these estimates, despite differences in laboratories and mouse strains, is further evidence that ESTRs are robust markers for studying germline mutation induction. In addition, our independent confirmation of the similarity between doubling doses for ESTR mutations, and those estimated for coding regions of the genome based on phenotypic markers (average 0.35 Gy, range 0.17 to 0.56 Gy, [4]) provides further support for a relationship between mutation rates at ESTR loci and those that affect phenotype.

Males in the adapted group (0.1 + 1.0 Gy) received the highest total radiation dose in our study, but their offspring inherited ESTR mutations at lower frequencies than those in the 0.5 or 1.0 Gy groups. This pattern, where a small prior radiation dose attenuates the effects of a subsequent larger dose, may be indicative of an adaptive response [13, 45,

46]. Our findings provide evidence that the protective effects of adaptive response in the mammalian germline may extend beyond gross chromosomal abnormalities in germ cells [35, 36], or dominant lethal mutations [37], and reduce the risk of transmissible genetic mutations to viable offspring.

The cellular mechanism for an adaptive response affecting germline ESTR mutation rates is unknown. The most commonly accepted explanation for chromosomal abnormalities is that DNA double strand breaks caused by the adapting dose induce an efficient DNA repair system that remains activated to provide additional repair following damage induced by the larger challenge dose [21]. Given that mutation induction at ESTR loci is not the result of direct physical damage to the repeat arrays themselves [4, 5, 6, 47], increased DNA repair at these sites likely does not explain the reduction in mutation frequency that we observed. We suggest that alternate pathways affecting genomic instability and / or apoptosis in affected germ cells must also be involved. In general, however, studies of ESTR mutation induction in mice following treatment with either radiation [4, 5, 12, 43] or chemical mutagens [48, 44] have shown remarkable parallels to those using more traditional genetic markers. In this case our findings are very similar to those of earlier *in vivo* studies on chromosome abnormalities in male mouse germ cells [35 - 37], and we therefore postulate that there may be a relationship between the adaptive responses observed using different genetic endpoints.

At this point it is not clear whether our findings apply to risk assessment for heritable mutations in humans exposed to ionizing radiation, but there are some interesting possibilities. For example, May *et al.* [49] did not detect an elevation in minisatellite mutation rate in the sperm of radiotherapy patients treated for seminoma. In

this study non-target testes received scattered radiation doses totalling 0.38 to 0.82 Gy – a range above the acute doubling dose for ESTR mutation induction in mice [4, this study]. A major difference from the mouse exposures in this case, however, was that the doses for radiotherapy patients were divided into 25 fractions delivered over a 21-day period. This would have resulted in testicular doses of 0.025 to 0.054 Gy approximately every 36 hours, perhaps resulting in the continuous induction of adaptive response-like pathways that reduced mutation induction. A similar explanation may also be plausible for the Chernobyl liquidators, who were exposed to fractionated radiation doses during clean-up following the reactor accident, but did not show elevated germline minisatellite mutation rates [50]. Further study of the effects of dose fractionation, dose rate, and adaptive response on germline mutation rates at repetitive DNA loci is required to test this hypothesis, and to provide more information about potential mechanisms.

In addition, because of the uncertain nature of the relationship between mutation rates at ESTR loci and those affecting coding regions of the genome, any influence on health risks conveyed by the adaptive response characterized here is unknown. Similar to other adaptive response studies, our results show that dose fractionation and timing may be more important determinants of the amount of genetic damage sustained than absolute radiation dose. A shortcoming of our study, however, is that we did not treat mice with the 0.1Gy-adapting dose alone, which may have provided some insight into the issues of radio-sensitivity and mechanisms of adaptive response discussed above. At the current time we conclude that there is evidence for an adaptive response based on the apparent suppression of germline mutations in males treated with 0.1 Gy followed by 1.0 Gy 24 hours later, compared to the group that received a single dose of 1.0 Gy. More research

on adaptive response and ESTR mutation induction is certainly required, but we do not recommend further experimentation with out-bred Swiss-Webster mice from the supplier used in this study. Adult mice often did not have alleles at ESTR locus *Ms6-hm* that were suitable for mutation detection using Southern blotting, resulting in double the number of DNA profiles required (and associated costs), and reducing the number of pups and bands that could be screened for mutations. Out-bred Swiss-Webster mice from other suppliers [38, C. M. Somers, *unpublished data*], or other mouse strains that have been tested for suitability of *Ms6-hm* and *Hm-2* allele sizes would be more efficient experimental systems.

Repetitive DNA markers have been used effectively to detect radiation-induced heritable mutations in the laboratory using mice [4, 43], and in natural populations of humans, birds, and plants living near radioactive contamination sites [51 - 55]. The potential value of this class of genetic marker for health risk assessment in radiobiology has recently been recognized [*e.g.*, 56]; however, little is known about the utility of this approach for the study of biological processes that modify risk, such as adaptive response, hormesis, or bystander effect (for an exception see multigenerational mouse studies that show a bystander effect that transcends generations [42, 57]). Our results suggest that adaptive response may reduce the frequency of mutation events at ESTR loci in the mammalian germline, and that ESTR markers are a suitable genetic system for further investigation of biological radiation risk modifiers. The relationship between repetitive DNA mutation rates and overall radiation risk to affected organisms, however, remains undetermined.

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Table 1. Descriptive data and statistical summaries for analysis of overall germline mutation rates in four groups of mice where males were treated with different doses of ionizing radiation from a cesium-137 source (see Fig.1 for mutation rates).

Treatment	Probe	Litters ^a	Pups ^a	Bands scored ^a	Mutant bands	Ratio to Control	P-value ^b	Bonferroni Corrected P-value ^c
Control	<i>Ms6-hm</i>	7	42	77	4			
	<i>Hm-2</i>	10	58	116	9			
	Single			193	13			
0.5 Gy	MMS10	10	58	847	12			
	<i>Ms6-hm</i>	7	39	85	14			
	<i>Hm-2</i>	10	56	108	18			
	Single			193	32	2.48	0.006	0.036
	MMS10	10	56	803	24	2.14	0.028	0.084
1.0 Gy	<i>Ms6-hm</i>	8	42	70	12			
	<i>Hm-2</i>	7	38	72	13			
	Single			142	25	2.63	0.006	0.030
	MMS10	8	47	673	24	2.57	0.008	0.032
0.1 + 1.0 Gy	<i>Ms6-hm</i>	8	44	79	7			
	<i>Hm-2</i>	9	52	111	15			
	Single			190	22	1.73	0.092	0.184
	MMS10	9	52	793	11	1.00	0.660	0.660

^aThe outbred Swiss-Webster mice we used had a wide range of *Ms6-hm* and *Hm-2* fragment sizes. A number of parents had only 1 or 0 detectable alleles at each of these loci, resulting in a variable number of litters, pups, and bands assayed by each probe within groups. In addition, in several families ESTR single locus probes detected more than 2 high-intensity bands in the profiles of both parents and offspring. These bands

were included in mutation scoring when somatic mosaicism in pups had been ruled out as the possible origin of extra bands.

^bProbability value for a two-tailed Fisher's Exact test comparing irradiated groups to the control.

^cSequential Bonferroni-corrected probability value for two-tailed Fisher's Exact test comparing irradiated groups to the control. The P-values have been corrected for 6 pairwise comparisons.

Table 2. Descriptive data and statistical summaries for analysis of paternal and maternal germline mutation rates in four groups of mice where males were treated with ionizing radiation from a cesium-137 source. Data presented are for single locus ESTR probes *Ms6-hm* and *Hm-2* combined (footnotes as in Table 1; see Fig. 2 for mutation rates).

Group	Bands scored ^a	Mutant bands	Ratio to Control	P-value ^b	Bonferroni Corrected P-value ^c
PATERNAL					
Control	91	8			
0.5 Gy	100	25	2.84	0.012	0.036
1.0 Gy	75	20	3.03	0.012	0.024
0.1 + 1.0 Gy	103	15	1.66	0.194	0.194
MATERNAL					
Control	102	5			
0.5 Gy	93	7	1.53	0.364	n/a
1.0 Gy	67	5	1.53	0.412	n/a
0.1 + 1.0 Gy	87	7	1.63	0.336	n/a

^cSequential Bonferroni-corrected probability value for two-tailed Fisher's Exact Test comparing irradiated groups to the control. Corrected P-values are based on 3 pair-wise comparisons within the paternal category as presented.

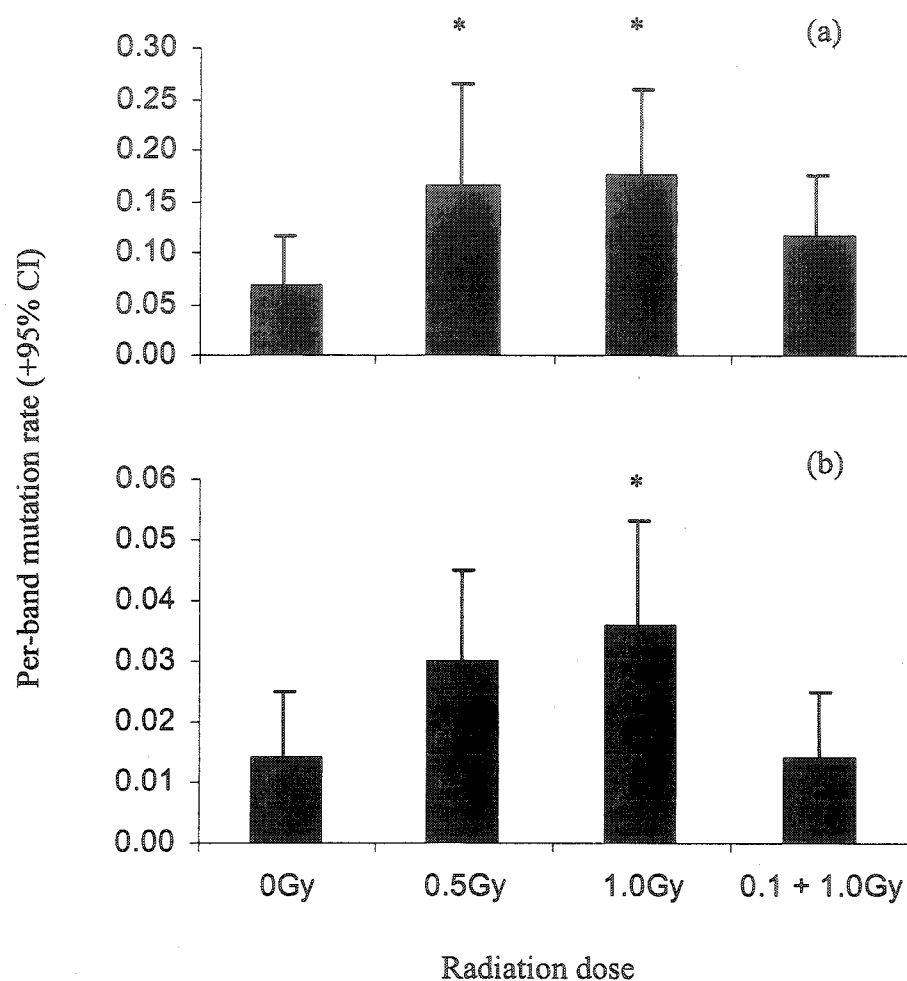


Fig 1. Overall germline mutation rates at ESTR loci in groups of mice where males were treated with four different doses of ionizing radiation from a cesium-137 source. Mutation rates were determined using (a) single locus markers *Ms6-hm* and *Hm-2* pooled, and (b) multilocus probe MMS10. Asterisks indicate a significant difference from the control group after Bonferroni correction as described in Table 1.

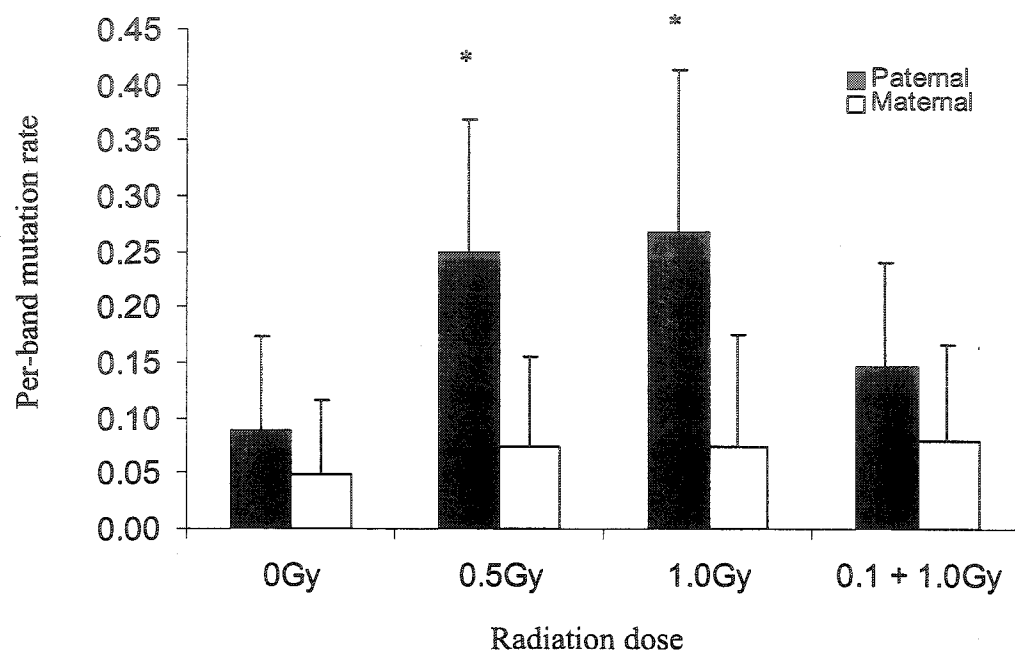


Fig 2. Paternal and maternal germline mutation rates at ESTR loci in four groups of mice where males were treated with ionizing radiation from a cesium-137 source. Mutation rates are for ESTR single loci *Ms6-hm* and *Hm-2* pooled. Asterisks indicate a significant difference from the control group after Bonferroni correction; details on statistical comparisons are described in Table 2.

CHAPTER 7

Synthesis and Recommendations for Future Research

7.1 The Route of Mutagen Exposure

One of the main questions I set out to address in this thesis was whether air pollution exposure, or a diet of contaminated fish was more important for inducing germline mutations in organisms living in the Hamilton Harbour area. This was an important question outstanding from previous studies of herring gulls (Yauk and Quinn 1996, Yauk et al. 2000) because each route of exposure carries a different level of potential risk for humans and wildlife. Air pollution from integrated steel mills and traffic has the potential to travel long distances and can affect many other species, including humans living or working nearby. However, humans do not subsist on a diet of fish harvested from waters near steel mills, particularly small-bodied fish species common in the diet of piscivorous birds like herring gulls. So a contaminated fish diet is likely to pose a risk to fewer organisms, but may still be important for piscivorous wildlife in polluted areas.

Chapters 2 and 3 describe significant elevations in the germline ESTR mutation rates of mice exposed to ambient air in Hamilton Harbour, compared to those exposed at a rural site removed from point sources of pollution. In both cases the elevation was due largely to increases in the frequency of mutant fragments passed to offspring through the paternal germline. These findings clearly demonstrate that ambient urban-industrial air pollution exposure alone was capable of causing elevated germline mutation rates in mice. Further, in Chapter 3 HEPA-filtration of ambient air significantly reduced germline mutation rates in mice exposed at the Hamilton Harbour site, highlighting the importance of airborne particulate matter in the induction of the observed genetic effects. Previous

studies of DNA damage in human sperm have suggested the possibility of air pollution-induced germ cell mutations (e.g., Perreault et al. 2000, Selevan et al. 2000), but were unable to determine whether genetic damage translated into transmissible mutations, nor could they attribute DNA damage solely to air pollution. Air pollution was also implicated in previous studies of germline mutations in herring gulls, but other contributing factors could not be ruled out (Yauk et al. 2000). The findings from sentinel animal studies presented here are the first to show directly that air pollution can indeed induce heritable mutations.

Chapter 5 describes a non-significant elevation in the germline ESTR mutation rates of mice fed a diet of smelt and alewife from Hamilton Harbour, compared to those fed either commercial dry chow, or clean fish from Atlantic Canada. This study was partially confounded by a spontaneous lymphoma outbreak that reduced the sample size of pedigrees available for DNA profiling, and thus reduced statistical detection power for comparisons among treatment groups. Sample size calculations showed that at least double the number of pedigrees would have to be screened to show a significant difference in mutation rates between groups of mice fed fish from Hamilton Harbour, compared to those eating fish from Atlantic Canada. In addition, statistical analyses that incorporated among-family variance in mutation rates showed no trend towards an elevation in mice fed fish from Hamilton Harbour. The combination of small sample sizes and high variance in mutation rates in this study therefore do not permit a firm conclusion regarding germline ESTR mutation induction resulting from consumption of fish common in the diet of herring gulls. The high levels of organic contaminants detected in the tissue of smelt and alewife, and the trend for paternal mutation rates to be

higher in mice fed fish from Hamilton Harbour, indicate that the dietary route of mutagen exposure may contribute to induced mutations in wildlife (e.g., herring gulls or other piscivores).

7.2 Health Implications

My findings indicate that humans and other animals living in areas with high levels of particulate air pollution may be exposed to germ cell mutagens through the simple act of breathing. The consequences of such exposures for future generations are currently unknown. This uncertainty stems in part from the fact that the relationship between mutation rates at ESTR loci and those in coding regions of the genome remains uncharacterized. Previous studies of ionizing radiation in mice have shown that ESTR mutation rates increase in a dose-dependent manner, and that the doubling dose for ESTR mutation induction is very similar to that determined using mouse phenotypic markers in the specific locus test (Dubrova et al. 1998). The results of the radiation exposure described in Chapter 6 provide a similar estimate of doubling dose. These radiation studies provide indirect evidence for a correlation between mutation rates at ESTR loci and those in coding regions that may affect the phenotype, and ultimately the health of offspring. Consequently, the offspring of organisms exposed to high levels of particulate air pollution in urban-industrial areas may be more likely to suffer from a wide range of inherited genetic disorders.

In considering the possibility for health effects caused by a general increase in mutations induced in coding regions of the genome, it should be emphasized that little is known about *why* the radiation doubling dose for ESTR mutation induction and that for

coding regions is similar. ESTR mutations occur at much higher rates than expected (up to 100-times more) following ionizing radiation exposure, suggesting a mechanism for mutation induction that does not involve direct damage to the repeat arrays themselves (Bridges 2001). This phenomenon is well outside the normal paradigm for mutations induced in coding loci, which typically result from very localized DNA damage in the affected region (Yauk 2004). Without more insight into the mechanism of ESTR mutation induction, it is not possible to exclude coincidence as a possible explanation for the similarity between the radiation doubling-dose estimates. In addition, the induction of ESTR mutations by chemical mutagens, as proposed for air pollution, has not been well studied (for examples see Hedenskog et al. 1997, Vilarino-Guell et al. 2003). Chemicals seem to induce ESTR mutations through an indirect mechanism, similar to radiation, but again the relationship to mutation events in coding regions is unknown (Bridges 2003, Yauk 2004).

Even if ESTR mutations are not an accurate reflection of what is happening at coding regions, mutations in tandem repeat sequences may themselves pose health risks to future generations. For example, humans with mutant alleles of a minisatellite flanking the H-ras1 proto-oncogene are much more susceptible to breast, colorectal, and bladder cancer, as well as leukemia (Krontiris et al. 1993). In addition, fetuses with mutant minisatellite alleles flanking the H-ras1 locus were significantly more likely to be spontaneously aborted (Kiaris et al. 1995). Inherited mutant alleles in minisatellites are also linked to development of type-1 diabetes (Bennett et al. 1995, Kennedy et al. 1995) and myoclonus epilepsy (Virtaneva et al. 1997). Size-change mutations in DNA elements with shorter core sequences (e.g., tri-nucleotide repeats) have been shown to cause a suite

of inherited neuro-degenerative disorders (Jaworski et al. 1995), including Huntington's disease (Yoon et al. 2003). If my findings in sentinel mice apply to humans, exposure to high levels of air pollution may be causing mutations in tandem-repeat arrays to be passed to subsequent generations at a higher frequency, thus increasing the risk of developing any of the above (or other) conditions. No studies to date, however, have attempted to link environmental contaminant exposure with mutations in the specific tandem-repeat arrays described above.

7.3 Air Quality: Comparing Hamilton Harbour to Other Locations

Based on the findings presented in Chapters 2 and 3, it is of considerable interest to compare air quality at the Hamilton Harbour mouse exposure site to that in other cities in Canada, and also the rest of the world. Data on total suspended particulate (TSP) and polycyclic aromatic hydrocarbon (PAH) levels at the Hamilton Harbour site are presented in Chapter 3. These analyses showed TSP ranging from 40 to 127 $\mu\text{g}/\text{m}^3$, and total PAH from 3 to 27 ng/m^3 based on wind direction. Sanderson et al. (2004) recently reported on particulate-associated PAH levels in Canadian cities (but not TSP). In general, other cities in Canada have much lower PAH concentrations than we measured in Hamilton Harbour. For example, Edmonton, Alberta had an average PAH concentration of 2.8 ng/m^3 , Windsor, Ontario 4.2 ng/m^3 , and downtown Hamilton, upwind of the steel mills had a PAH concentration of 4.2 ng/m^3 . So average PAH levels measured at our study site (13.4 ng/m^3) were on average 3.2-times as high as those in other Canadian urban environments, but almost 5-times lower than the highest in Canada (66.2 ng/m^3 ; Jonquiere, Quebec), measured 1.5 km downwind from a large aluminium smelter.

Average particulate-associated PAH levels in American cities are also considerably lower than those measured in our study. Los Angeles, California had PAH levels of 1 to 2 ng/m³, Houston, Texas, 0.5 to 1.0 ng/m³, and Elizabeth, New Jersey, 3.5 to 4.5 ng/m³ (Naumova et al. 2002). In contrast, some European and Asian cities have PAH levels similar to, or even much higher than those in Hamilton Harbour. For example, in Stockholm, Sweden, PAH levels ranged from 8 to 25 ng/m³ (Bostrom et al. 2002), and in Bangkok, Thailand, average particulate-associated PAH levels were 60 ng/m³ (Thongsanit et al. 2003). So it is clear from these comparisons that concentrations of PAH in Hamilton Harbour are high by North American standards, but only intermediate on a global scale.

Sao Paulo City, Brazil, is the largest city in Latin America, and the only other location in the world where sentinel mice have been exposed to ambient air pollution in a genotoxicity study. Soares et al. (2003) detected significantly elevated frequencies of micronucleus formation in the peripheral erythrocytes of mice exposed in downtown Sao Paulo, compared to others exposed 65 km away in a rural area. The average TSP level in Sao Paulo City was 122 ug/m³, which is at the high end of the range determined for Hamilton Harbour based on wind direction in Chapter 3. Particle-associated PAH levels in Sao Paulo, however, were considerably lower than those in Hamilton Harbour, with an average of 3.1 ng/m³ (Vasconcellos et al. 2003). The importance of particulate-associated PAHs for the genotoxic effects observed in sentinel animals remains undetermined; however, if PAHs are important for heritable mutation induction, then it is clear that the risk of induced effects will be high at other locations in world.

7.4 Recommendations for Preventing Particulate Air Pollution Effects

Airborne particulate matter played an important role in the induction of germline ESTR mutations in Chapter 3. Based on this finding alone, a sensible precautionary approach would be to take steps to reduce emissions of particulate matter from all sources in urban and industrial environments. However, the discovery of a trans-generational genetic effect induced by airborne particulate matter should not be considered in isolation. Large-scale human epidemiology studies have already linked particulate air pollution exposure to a variety of serious human health effects, including lung cancer (Cohen 2000, Arden Pope et al. 2002), cardiovascular and respiratory conditions (Delfino et al. 1994, Burnett et al. 1995, Yang et al. 2004), impaired fetal development (Dejmek et al. 1999), and even death (Schwartz 1994, Arden Pope et al. 1995, Brunekreef and Holgate 2002). Furthermore, we currently understand very little about how particulate air pollution causes these conditions, and what ambient levels represent 'safe' exposures (Krewski et al. 2003). It is therefore beyond contention that exposure to airborne particulate matter must be reduced.

One possible option for humans living in heavily impacted areas, such as near industry or busy roads, is the use of HEPA-filtration equipment. Operation of portable room-filtration systems can effectively reduce indoor particle levels (Rutala et al. 1995, Abraham 1999), but the efficacy of this approach for reducing health effects from air pollution remains uncertain. HEPA filtration was effective in preventing induction of germline mutations in Chapter 3, but the filter in this case was used as a barrier between ambient air and the sentinel mice. This level of protection would not be possible for any human residence, and would not address the issue of outdoor exposure, or effects on

wildlife. HEPA-filtration should therefore be viewed as a temporary protective measure in lieu of reducing particulate emissions at their source.

Particulate matter is emitted to the atmosphere from a variety of sources, but processes associated with the production of steel, and vehicle traffic are the two important sources for the effects observed in Chapters 2 and 3. Analysis of both TSP and PAH levels in Chapter 3 revealed a significant effect of wind direction. Specifically, both TSP and PAH levels were dramatically elevated at the Hamilton Harbour mouse exposure site when the wind was from the west, carrying with it particulate matter from the city of Hamilton and the two integrated steel mills. Comparatively low TSP and PAH levels on days when the mouse exposure site was upwind of the industrial area suggest that the steel industry was contributing a large component of the measured airborne emissions. A reduction in particulate emitted from the steel mills would be an obvious step towards reducing exposures at all surrounding locations.

Emissions from vehicles have also been implicated in causing human health effects, and will likely continue to make a large contribution to air pollution problems in cities (Burr et al. 2004, Hutchinson and Pearson 2004). Incentives for fuel-efficient vehicles, improved public transportation systems, and a reduction/discouragement of urban sprawl will aid in reducing the impact of cars and trucks on urban air quality. Adult trees have also been shown to reduce airborne particulate levels (Beckett et al. 1998), so city planning should include large green spaces with many trees.

7.5 Utility of Sentinel Mouse Exposures

Sentinel mouse exposures can provide clear and direct information on the genetic effects of exposure to ambient environmental conditions, as demonstrated in Chapters 2, 3, and 5. This approach provided valuable follow-up information to previous germline mutation studies in wildlife (Yauk and Quinn 1996, Yauk et al. 2000), and is potentially well suited for other applications, including further evaluation of air pollution induced effects, and experimental verification of perceived risks in areas close to major industrial developments. Overall, sentinel animal experiments have been under-utilized in terrestrial toxicology, and offer a great deal of promise for future studies of genotoxicity.

Endorsement of sentinel mouse exposures comes with two notes of caution. First, while biological responses to environmental exposure may be easier to measure in lab animals, extrapolating findings to other organisms remains difficult. Second, in mouse studies care should be taken in selecting the strain and supplier to be used. Some mouse strains respond differently to mutagen exposure (Bhilwade et al. 2004), and some animal suppliers have disease problems, such as the lymphoma outbreak described in Chapter 5. These factors might influence the interpretation of experimental results. For ESTR mutation studies, the size range of Ms6-hm and Hm-2 fragments in particular mouse strains and colonies is also an important consideration. For example, in Chapter 6 Ms6-hm fragments were too small to be detected in a large number of mice, reducing the number of bands that could be scored for mutations.

7.6 Radiation Dose Response

ESTR mutation rates in the paternal germline of the out-bred Swiss-Webster mice used in Chapter 6 showed a non-linear dose response to ionizing radiation. Nevertheless, the doubling-dose estimate of 0.26 Gy was very close to that determined for ESTRs and other markers in inbred strains (0.17 to 0.5 Gy; Dubrova et al. 1998). This is preliminary evidence that inbreeding/out-breeding *per se* does not fundamentally alter the response of mice to mutagen exposure. The genetic variability in out-bred mice presents some problems for detection of small ESTR fragments, and resolution of small mutations in very large alleles. Given the lack of apparent difference in ESTR mutation doubling dose between inbred and out-bred lines, it would make sense for future sentinel mouse studies to use previously characterized F1 hybrids between inbred strains (e.g., Yauk et al. 2002) for more consistent mutation scoring.

The suppression of ESTR mutations apparent in the group of mice receiving a small adapting dose of ionizing radiation prior to a larger challenge dose (Chapter 6) is evidence for an adaptive response in the paternal germline. This finding requires verification with a larger sample size, but raises questions regarding exposure scenarios that can induce adaptive response, and the possibility for similar processes occurring under chronic, low-level environmental exposure conditions. For example, are the elevated mutation rates induced by air pollution in Chapters 2 and 3 'normal', or are they already suppressed to some degree by adaptive response processes? More insight into the mechanisms of ESTR mutation induction and adaptive response is required to evaluate this issue.

7.7 Directions for Future Research

More questions than answers have been generated over the course of conducting the research for this thesis. Below are listed some directions for future research that I feel will be important in advancing our knowledge in this area of genetic toxicology.

Determination of exposure scenarios that cause mutation induction - Particulate air pollution was required to significantly induce germline ESTR mutations in Chapter 3, but the exposure levels and chemical composition of particulate required to cause these genetic changes are unknown. In addition, there was evidence for a possible effect of dietary contaminant exposure in Chapter 5. Future research should attempt to determine what kinds of environmental exposures are capable of causing significant mutation induction.

Identification of the link between lung exposure and ESTR germline mutations – In Chapters 2 and 3, air pollution exposure caused elevated ESTR mutation rates predominantly in the paternal germline, indicating that contaminant exposure through the lungs was capable of affecting developing spermatogonial cells in the testes. Controlled laboratory experiments to confirm this pathway will be important.

Characterization of ESTR mutation processes in the female germline – In Chapters 2 and 3, slight (non-significant) elevations in maternal mutation rates were detected in the mice exposed to air pollution at the urban-industrial site in Hamilton Harbour. Little is understood about the induction of ESTR mutations in female germ cells. Laboratory and environmental exposures to characterize ESTR mutation induction processes (timing, sensitivity to known mutagens, etc.) in females are required.

Establishing the relationship between ESTR mutation rates and those in coding regions of the genome – Understanding how contaminant exposure induces ESTR mutations, and the relationship between ESTR mutation rates and those that affect phenotype, and ultimately health, will be crucial in linking this biomarker to more meaningful biological effects. Controlled lab studies examining ESTR mutations and a suite of other more functional markers simultaneously will be required.

Expansion of sentinel animal exposures to other sites – Hamilton Harbour is the only location where mice have been exposed to air pollution for the purpose of studying germline mutations. Although herring gulls have been studied at other industrial locations (Yauk et al. 2000), it is necessary to use mice to test other sites and other sources of air pollution to determine how widespread and general the genetic effects of air pollution are.

Human epidemiology studies – Sentinel mouse exposures do not permit direct inference regarding potential health consequences for humans. Epidemiology studies that investigate the possibility of a relationship between air pollution exposure and inherited diseases would be a logical and pertinent follow-up to sentinel mouse studies. Given the nature of mutations in herring gull and sentinel mouse studies, diseases caused by mutations in repetitive DNA elements would be an interesting starting point for making a direct human health connection.

Pedigree DNA profiling studies in humans – Comparisons of minisatellite mutation rates in human families living in high and low air pollution areas are necessary to determine whether human tandem-repetitive DNA responds similarly to that of mice. In addition, the same approach could be used to study mutation rates in families with high

parental occupational exposure to chemical mutagens, such as coke oven workers. These studies would not provide the same potential clarity as sentinel animal exposures, but would be directly on human subjects.

Germline mutation studies in additional wildlife species – ESTR-like DNA elements with high spontaneous mutation rates have been characterized in several other species, including lizards (Tokarskaya et al. 2004) and birds (Lubjuhn et al. 2002). Development of similar markers for more wildlife taxa, and the application of germline mutation screening in a broader range of species will provide considerable insight into the general phenomenon of repetitive DNA mutations. Availability of markers for a range of taxa would also allow screening for induced mutations in organisms exposed to a wide variety of very different environmental conditions.

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MAIN APPENDIX 1

EXAMPLES OF ESTR MUTATIONS

Examples of ESTR mutations detected with single locus probe Hm-2 are provided in Chapter 2. In this appendix I show examples of mutant bands detected using the other single locus probe, Ms6-hm, and also ESTR multilocus probe MMS10. The pedigrees shown are from the air pollution exposures described in Chapter 3, but are representative of the kinds of mutation events detected in all of my experiments.

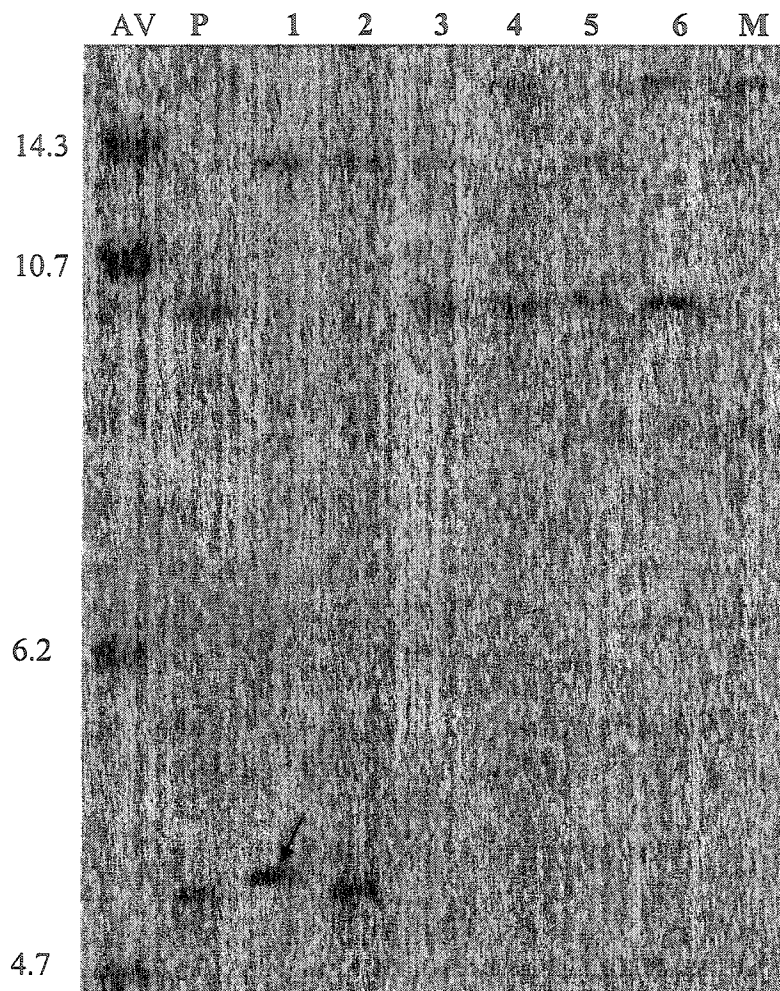


Fig 1. Autoradiograph of a mouse pedigree profiled using single locus ESTR marker Ms6-hm. The outside lanes show the paternal (P) and maternal (M) profiles, and lanes 1 through 6 are their offspring. The arrow highlights a mutant band of paternal origin in pup 1. The size range in kilobases is indicated on the left of the figure, corresponding to BamHI-digested adenovirus DNA (AV).

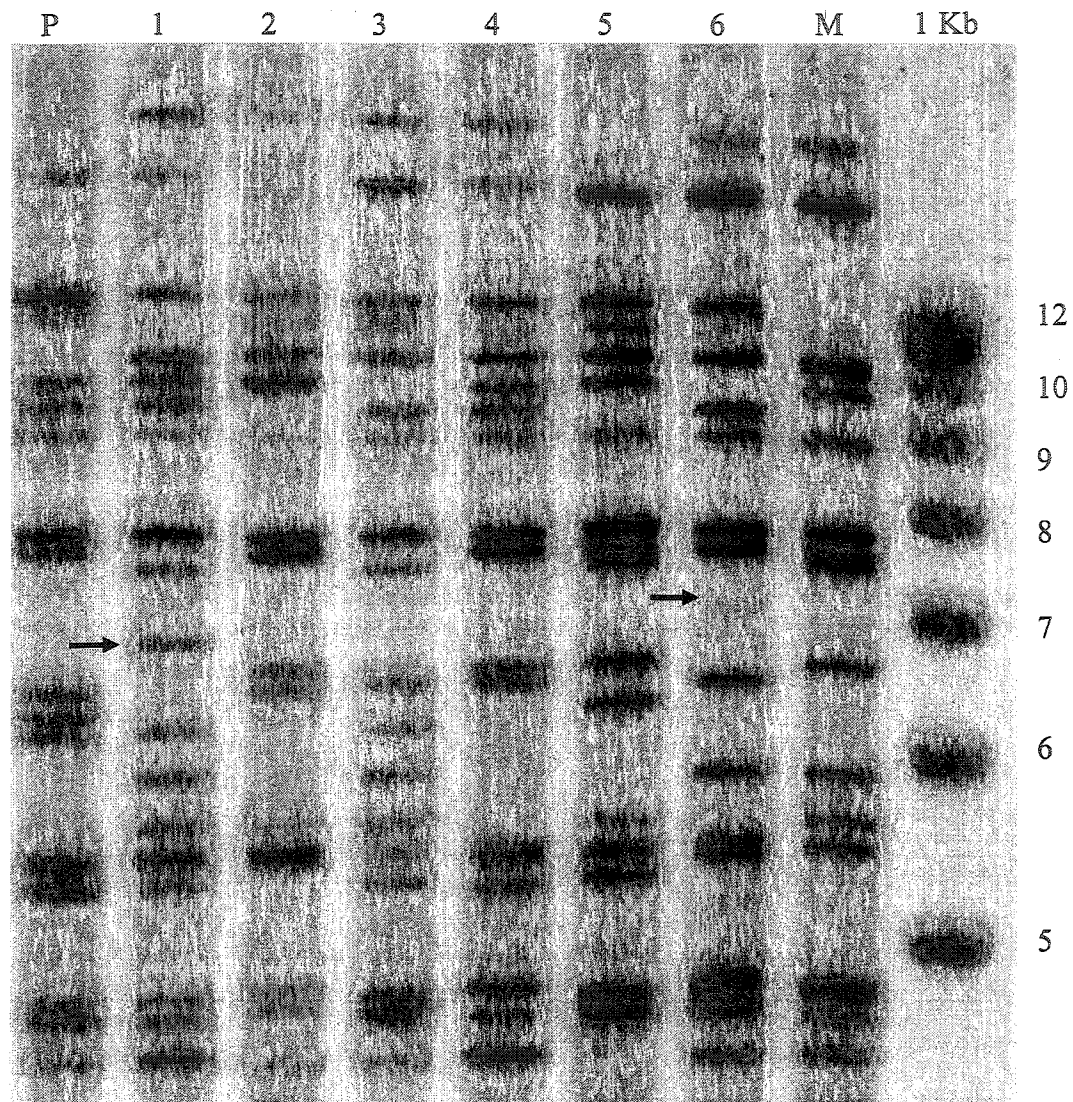


Fig 2. Autoradiograph of a mouse pedigree profiled using multilocus ESTR probe MMS10. The outside lanes show the paternal (P) and maternal (M) profiles, and lanes 1 through 6 are their offspring. The arrows highlight mutant bands in offspring 1 and 6. The size range in kilobases is indicated on the right of the figure based on a commercial 1 kilobase DNA ladder (1 Kb).