

DNA MUTATIONS IN WILD HAMILTON HARBOUR CORMORANTS

MICROSATELLITE DNA MUTATIONS AND POLYCYCLIC AROMATIC HYDROCARBON
(PAH) METABOLITES IN WILD DOUBLE-CRESTED CORMORANTS (*PHALACROCORAX
AURITUS*) FROM HAMILTON HARBOUR ASSOCIATED WITH EXPOSURE TO AIRBORNE
POLLUTANTS

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TITLE: Microsatellite DNA mutations and Polycyclic Aromatic Hydrocarbon (PAH) metabolites in wild Double-crested Cormorants (*Phalacrocorax auritus*) from Hamilton Harbour associated with exposure to airborne pollutants

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Abstract

Hamilton Harbour is one of the most polluted sites on the Great Lakes, affected by airborne and sedimentary contamination as a result of both heavy vehicle traffic and thousands of kilograms of industrial steel emissions. Polycyclic Aromatic Hydrocarbons (PAHs) are ubiquitous mutagenic byproducts of incomplete organic combustion; they are present at very high concentrations in the air and sediment of Hamilton Harbour. We quantified DNA mutation rates in three different nesting colonies of Double-crested Cormorants (*Phalacrocorax auritus*) using five microsatellite markers. These colonies were located at various distances from sources of PAHs and other contamination. We compared pollution-exposed and reference colonies, hypothesizing that cormorants living closest to pollution will have higher rates of germline microsatellite mutations than those living farther away from pollution sources. Using a pedigree approach, we identified mutations when chicks showed microsatellite alleles not found in either parent, and other explanations such as extra-pair parentage had been ruled out. Microsatellite mutation rates were 4.4 times higher at the Hamilton Harbour site closest to the industrial sources of PAH contamination than the other Hamilton Harbour site, and both were higher than the reference colony. Metabolites of the PAH benzo[a]pyrene in cormorant tissues from both Hamilton Harbour sites were identified by LC-MS/MS, demonstrating that cormorants in Hamilton Harbour are exposed to, and metabolizing, PAHs. Diet was not substantially different between the two Hamilton Harbour colonies when measured with regurgitated samples and fatty acid analysis. This suggests airborne pollution in Hamilton Harbour induced germline mutations in cormorants.

Acknowledgements & Preface

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List of all Abbreviations and Symbols

AOC: Area of Concern

bp: base pair (of DNA)

DFA: Discriminant Function Analysis

ESTR: Expanded Simple Tandem Repeat

HAMN: Hamilton Air Monitoring Network

IARC: International Agency for Research on Cancer

LC-MS/MS: Liquid Chromatography-Tandem Mass Spectrometry

MUFA: Monounsaturated Fatty Acid

NPRI: National Pollutant Release Inventory

NPAH: Nitrogenated Polycyclic Aromatic Hydrocarbon

PAH: Polycyclic Aromatic Hydrocarbon

PCA: Principal Components Analysis

PCR: Polymerase Chain Reaction

PMI: Polymethylene-interrupted (fatty acid)

PUFA: Polyunsaturated fatty acid

QEW: Queen Elizabeth Way highway

RAP: Remedial Action Plan

SAFA: Saturated Fatty Acid

Chapter One: General Introduction

The health effects of chemical pollutants are a pressing concern. Modern anthropogenic developments such as industrial, agricultural, municipal, and consumer practices release contaminants into the natural environment. Both humans and wildlife can be exposed to toxic substances and their mixtures with deleterious effects on health. It is important to understand short-term and long-term health effects of these chronic threats, especially since chemical exposure can cause infertility and disease, as well as promote early death. Understanding the genetic impacts of chemicals to which we are continually exposed is therefore necessary. Furthermore, there is an urgent need to evaluate the health of wildlife populations in rapidly changing environments, often subject to chemical stressors (Acevedo-Whitehouse & Duffus 2009); the contaminated ecosystem of Hamilton Harbour provides a natural laboratory for ecotoxicology research.

Hamilton Harbour Emissions and Sediments

Hamilton Harbour, a 2150 hectare embayment at the west end of Lake Ontario, is one of the most polluted areas on the Great Lakes (Hall et al. 2006; IJC 1999), partially due to its history as a centre of industrial steel production since the 1800s. Toxic substances are the root cause of multiple problems in the Harbour, including degraded fish and wildlife populations and sedimentary contamination (Environment Canada 2010, Brousseau and Randall 2008). Designated as an Area of Concern (AOC) in 1987, a Remedial Action Plan drafted in 1992 specifies actions necessary to clean up the Harbour, including containment of

the Randle Reef area which is highly contaminated with Polycyclic Aromatic Hydrocarbons (PAHs) (Hall et al. 2006).

PAHs are a group of over 100 related hydrocarbon chemicals that are environmentally ubiquitous and can be detected almost everywhere in both indoor and outdoor air (Liu et al. 2001, Ohura et al. 2004, Naumova et al. 2002). They are produced mainly from human activities such as combustion of fossil and bio-fuels (including in vehicles), coke and asphalt production (and so in integrated steel production due to coke production), aluminum production, and waste incineration (Srogi 2007). Forest fires and natural sources such as volcanic eruptions also contribute PAHs to atmosphere and sediments (Sofowote et al. 2011), but in smaller amounts relative to anthropogenic sources (Zhang & Tao 2009). They are known *in vitro* mutagens (Quillardet et al. 1982), and many are carcinogenic to humans and animals. PAHs can have 3 to more than 15 carbon rings, but 5 or 6 ring compounds such as benzo[a]pyrene and dibenzo[a,l]pyrene have shown the highest mutagenic and carcinogenic activity (Müller et al. 2001; Layshock 2010). Additional effects of PAH exposure are numerous and in humans can include shortened telomeres (Pavanello et al. 2010), fragmented DNA in sperm (Rubes et al. 2007), decreased sperm competence (Mukhopadhyay et al. 2010), endocrine disruption, alterations in physiology and gene expression and various other types of DNA and chromosomal damage (Belpomme et al. 2007). Most PAHs are actively metabolized (biotransformed) quickly in many groups of organisms, 'bioactivating' them to reactive Phase I metabolites (hydroxylated or hydroxy-PAHs) that then exert toxic effects (Baird et al. 2005).

Emissions from industry, including Canada's two largest integrated steel mills, and diesel and gasoline emissions from nearby highways, are major contributors to the pollution in Hamilton Harbour (Sofowote et al. 2008), including very high PAH concentrations in air (Somers et al. 2004) and sediment (Zeman & Patterson 2003). Hamilton Harbour area industries emit thousands of kilograms of PAHs each year (e.g. NPRI 2009, 2010) including those carcinogenic to humans (e.g. benzo[a]pyrene, Table 1). Balch et al. (1995) demonstrated carcinogenicity to rainbow trout with Hamilton Harbour sediment samples, noting that PAHs and additional compounds were responsible for mutagenicity and carcinogenicity. McCarthy et al. (2004) identified PAHs as causing observed toxicity to four aquatic species in bioassays that used Hamilton Harbour sediment. Water movements in Hamilton Harbour allow highly contaminated historical buried sediments to be re-exposed (Zeman & Patterson 2003), such as the heavily contaminated Randle Reef site which continually releases PAHs into cleaner areas of the Harbour (Marvin et al. 2000). Therefore, even with current reduced inputs of PAHs to the Harbour, contamination will continue to be a problem into the future until remediation is completed.

PAHs in Hamilton Harbour Air – Synergistic considerations

PAHs emitted by industry and vehicles react with other substances and are transformed into mixed compounds, such as nitropolycyclic aromatic hydrocarbons (NPAHs) and oxygenated polycyclic aromatic hydrocarbons (oxy-PAHs) (Purohit & Basu 2000, Stiborová et al. 2009). Benzo[a]pyrene is one of the most potent mutagens of the PAHs; however, when it reacts with the nitrogen dioxide normally present in polluted air, the resulting

product nitrobenzo[a]pyrene (an NPAH) shows even higher mutagenic activity (Tokiwa et al. 1994). Oxy-PAHs have attached carbonylic oxygen; they are toxic to humans and animals, and show high persistence and mobility in the environment, which might increase their bioavailability (Lundstedt et al. 2007). An examination of air pollution in Beijing showed NPAHs and oxy-PAHs to be responsible for twice the mutation induction of PAHs in Ames assays, despite their presence in small amounts within the samples (Wang et al. 2011). Additionally, some PAHs are photomutagenic, meaning their mutagenicity can be increased or induced by ultra-violet (UV) light (Platt et al. 2008). PAHs in combination with light can cause single-stranded breaks, DNA adducts, and often at higher rates than either alone (Toyooka & Ibuki 2007), a concern as many environmental PAHs are exposed to sunlight. Mutations induced in Hamilton Harbour may therefore result from a wide variety of these mixed PAH compounds and their synergistic effects, and not just 'ordinary' PAHs. These compounds cannot be ignored when evaluating the mutagenic potential of Hamilton Harbour air, although they are not currently monitored.

This complex mixture of chemicals which forms Hamilton Harbour air pollution was suspected to have caused elevated germline DNA mutation rates in families of herring gulls (*Larus argentatus*) (Yauk & Quinn 1996; Yauk et al. 2000) and increased mutation rates in families of ambiently exposed mice (*Mus musculus*, Somers et al. 2002, 2004). Somers et al. (2004) demonstrated that high-efficiency particulate-air (HEPA) filtration, removing the majority of breathable particulates and PAHs from air, significantly decreased mutations in mice relative to mice at the same site that were breathing unfiltered air. Therefore, this study showed that particulate matter in polluted air induced, or caused, mutations, and that PAHs

probably played an important role in mutation induction.

Induced Mutations

Mutations are the preliminary stage of many diseases, including cancer (Frank 2010, Irigaray & Belpomme 2010). Many environmental chemicals are mutagens, capable of inducing various types of mutations in DNA. Metabolites of PAHs become covalently bonded to nucleotide bases in DNA, having a higher affinity for purine bases G (guanine) and A (adenine) (Pleasance et al. 2010). They then undergo further transformation to PAH-DNA adducts, which disrupt replication (Volk et al. 2003) and produce mutations when DNA replication proceeds using strands with unrepaired lesions (Lagerqvist et al. 2008). These subsequent mutations are the first step in cell transformation leading to PAH-caused cancers (Volk et al. 2003). Mutations can be induced in non-coding regions (including promoter regions), or coding regions, such as in the important p53 antitumour gene (Pavanello et al. 2010). An increase in mutations alone is cause for concern, but mutations could also be a signal of wider DNA damage as well, such as strand breaks and chromosomal changes (Yauk et al. 2008; Fox et al. 2005)

Mutations can be efficiently measured with tandem repeat DNA, which are stretches of repeated DNA sequences such as minisatellites and microsatellites. By using these types of DNA, it is possible to avoid sampling the extremely high number of individuals required to observe mutation induction if screening using coding regions under selection (e.g. Dahl et al. (2001)). What exactly qualifies as a microsatellite is somewhat arbitrary (Sia et al. 1997), but

many authors use a definition of 2-6 base pair sections repeated at least 8 times. Microsatellites have been found non-randomly throughout all eukaryotic genomes examined thus far (Grover & Sharma 2011), and can be found as perfect or imperfect repeats, interrupted repeats, and complex/compound microsatellites. A perfect repeat is one pattern repeated multiple times, e.g. ATGATGATGATGATG (written as ATG_5), while an interrupted repeat has one or more base pairs intervening, e.g. ATGATGCCCATGATGATG (written as $ATG_2CCCATG_3$) where three C bases split the sequence of repeat units. Compound and complex repeat can take a variety of forms with two types of repeats either next to each other or split by intervening nucleotides. Microsatellites are thought to mutate directly through replication slippage, also known as polymerase slippage or slipped-strand mispairing (Ellegren 2000, Kruglyak et al. 1998). This usually results in intra-allelic gain or loss of repeats, often one or two units. This model of microsatellite mutation is termed the stepwise mutation model, and has been widely used in explaining observed mutations (e.g. Ortego et al. 2008; Beck 2003; Brohede et al. 2002), but there is evidence that single unit repeats do not dominate in all cases (Macdonald et al. 2011; Keirle et al. 2011). Additionally, direct sequencing has confirmed that microsatellites sometimes also show short insertions and deletions within the microsatellite (Ellis et al. 2011). These studies add to accumulating evidence that there are more complicated mechanisms involved in microsatellite mutation.

A new model for microsatellite mutation, the life cycle model (Buschiazzo & Gemmill 2006), may better explain their evolution and mutation. It posits that microsatellites are continually changing as they develop new alleles throughout their creation, expansion, contraction, and eventual death. It is possible that both of these models will be useful, since

not all microsatellites will necessarily mutate the same way, depending on characteristics such as length, location, and taxonomic group.

Most microsatellites are non-coding DNA, which raises the question of whether mutations in these regions produce any deleterious health effects. However, genetic damage in repeat loci could reflect global genomic instability (Yauk & Polyzos 2005; Yauk et al. 2008, Armour 2006), and this might be due to overall interference with important repair mechanisms, polymerases, or enzymes involved in DNA repair. Up to 30% of human genes have tandem repeats contained within their coding regions (exons) (Legendre et al. 2007) and in a recent survey of human tandem repeats, 8% were within coding sequences (Wren et al. 2000). Additionally, microsatellite mutations have recently been linked to a variety of human diseases, notably cancer, especially in some colorectal, gastric, and endometrial cancers (microsatellite instability, (Imai & Yamamoto 2008)), tumours related to Lynch syndrome (Woerner et al. 2010), and Ewing (bone) tumours (Guillon et al. 2009). Microsatellites are also involved in type 1 diabetes (insulin gene promoter, (Bois & Jeffreys 1999)), some muscular dystrophies, trinucleotide repeat diseases (e.g. Huntington's, fragile-X), epilepsy, other genetic diseases in the alphasynuclein family (Somers & Cooper 2009, Gomes-Pereira & Monckton 2006) and autism (microsatellites in flanking region of vasopressin receptor gene, Meyer-Lindenberg et al. 2009). The coming years will likely reveal more microsatellite-related health effects in humans and other species, further validating their use as a genetic biomarker.

Microsatellite Genotyping for Mutation Analysis

Microsatellites can be isolated from genomic DNA with relatively simple technology (e.g. St. John & Quinn (2008)) or quickly using next-generation sequencing technologies (Abdelkrim et al. 2009; Haig et al. 2011), allowing their application in non-model animal studies (Guichoux et al. 2011). They are also amplified with polymerase chain reaction (PCR), can be genotyped with automated DNA analyzers, and in most cases allow identification of the parent and allele that contributed the mutation. While minisatellites will likely continue to be useful, especially in humans (e.g. Dubrova et al. (2006)), the numerous logistical advantages of microsatellites have resulted in their recent widespread use.

Microsatellites can be used to evaluate germline mutagenicity (mutations inherited by offspring) by pedigree screening. In this technique, genotyping in both parents and chicks allows for quantification of genetic damage across generations (transgenerational effects) as opposed to simply somatic mutations. Using this approach, microsatellite mutation rates have been examined in Hamilton Harbour populations of fish and birds. Miller (2009) identified mutations in one of four microsatellite loci examined in fathead minnows (*Pimephales promelas*) exposed *ex situ* to sediment from highly PAH contaminated Randle Reef. Anmarkrud et al. (2011) compared mutation rates in one highly variable microsatellite marker in tree swallows (*Tachycineta bicolor*) from Windermere basin, in Hamilton Harbour, to those from a relatively unpolluted area of Eastern Ontario. Although both studies did detect a trend towards a slightly higher mutation rate in the contaminated group, neither was statistically significant. This highlights the difficulty of this type of research, in which sample sizes can be

relatively low and a number of confounding factors may be present. Various factors may have played a role in the lack of significant differences in mutation in these studies. It is possible that the species chosen were not ideal. Fish may be naturally more resilient to contamination (e.g. (Wirgin & Waldman 2004)), and it is possible that exposure via contaminated sediment may produce a smaller magnitude of effect than exposures that include airborne contamination routes as well. Other factors that could have limited induced mutations include timing of exposures, sample sizes, or the particulate microsatellite loci chosen. Microsatellites vary enormously in their mutability, and while both Miller and Anmarkrud et al. attempted to use variable markers, they simply may not have been variable enough to demonstrate statistically significant mutation induction (see also Chapter 4, Conclusion). This represents an area to improve on previous microsatellite mutation studies in Hamilton Harbour, by increasing the number and mutability of microsatellites used.

Cormorants as biological indicators of pollution

Double-crested Cormorants (*Phalacrocorax auritus* Lesson 1831) are a more suitable indicator species for a Hamilton Harbour microsatellite germline mutation screening study than the fathead minnows and tree swallows used previously. Fish-eating birds have been some of the earliest and most important indicators of contamination-related health effects in the environment (Foster & Wang 2011). Exposure to other chemicals that resulted in eggshell thinning and chick edema (Ludwig et al. 1996; Yamashita et al. 1993) raised the profile of cormorants and other waterbirds as indicator species. Both Double-crested and Great Cormorants (*P. carbo*) have previously been used and recommended as biological indicators

(Somers et al. 1993; Fox 2001, Dirksen et al. 1995).

Cormorants are large-bodied, with a high basal metabolism even relative to other birds their size (Hennemann 1988). With unidirectional air flow in the lungs and high lung volumes, birds inhale large amounts of particulate matter and could be 'valuable experimental models' for air pollution studies (Brown et al. 1997). Cormorants in Hamilton Harbour could also absorb genotoxins from contaminated water, soil, or sediment, and feed on a variety of aquatic species that are probably contaminated to different degrees. However, they eat almost entirely fish (Hatch & Weseloh 1999), and no human food waste, as do gulls. Therefore, they will pick up contamination only from natural food web sources. Cormorants are abundant in Hamilton Harbour, hardy, relatively easy to trap and sample, and large enough to collect appropriately sized blood samples for a number of analyses. They are also common enough in different areas of the lower Great Lakes to allow for the use of a reference site nearby.

Double-crested Cormorant diet has been a subject of intense interest for many years due to rapidly increasing populations in some areas (Weseloh & Ewins 1994) and awareness surrounding potential conflicts with recreational and commercial fisheries (Dorr et al. 2010; Burnett et al. 2002, Lantry et al. 2002), so much research has been done on the subject. This allows us to better understand possible dietary sources of PAH and PAH metabolite exposure in cormorants, so that dietary and airborne exposure can be contrasted. However, a more detailed understanding of diet differences and similarities among the three sites would be optimal, especially since zebra and quagga mussels (*Dreissena* spp.) and round gobies are

implicated in Great Lakes-wide contaminant transfer pathways (Kwon et al. 2006). Differences in diet that might affect mutation rate would be an important factor in untangling the complex sources of exposure that undoubtedly affect cormorants in Hamilton Harbour. Fatty acid analysis provides a useful aid in this goal, since they allow for a very fine resolution of diet similarities among the three sites within a time frame of approximately 1-2 months. In the cormorant food web, the various fish species consumed have unique fatty acid profiles (Czesny et al. 2011), obtained in turn from the fish's diet such as phytoplankton, zooplankton, detritus, or microbes (Smyntek et al. 2008; Rossi et al. 2006; Käkälä et al. 2009). Therefore, fatty acid profiles are largely conserved as they move upwards through food webs, to higher trophic level predators (Hebert et al. 2006, 2008) such as cormorants. The use of two sites within Hamilton Harbour combined with fatty acid analysis will help tease apart differences stemming from airborne and diet-based contaminant exposure, since fatty acid analysis can be used to demonstrate similar diets at the two colonies, implying airborne exposure as the main difference between them.

Linking mutations to PAH exposure in cormorants

Quantifying PAH metabolites in tissues will demonstrate that cormorants in Hamilton Harbour are exposed to PAHs, such that observed mutations could be caused by this exposure. A metabolite of the PAH pyrene, 1-hydroxypyrene, is commonly used as a biomarker of PAH exposure, with high concentrations in humans occupationally exposed to PAHs, such as firefighters (Edelman et al. 2003) and coke oven workers (Pavanello et al. 2010), as well as in high-exposure groups in the general population (Li et al. 2008). Though

most PAH metabolite research has been on humans, several hydroxylated PAHs have been detected in various samples and tissues in environmentally or experimentally contaminated animals (goat milk, blood, and urine: Grova et al. 2005, 2008, Lapole et al. 2007; cow, horse, rabbit, pig manure: Rey-Salgueiro et al. 2008; chicken eggs: Fournier et al. 2010). In oiled common guillemots (*Uria aalge*), the majority of PAH compounds were found as hydroxylated metabolites (often twice and up to seven times more metabolites than parent PAHs (Troisi et al. 2006). To our knowledge no previous study has shown PAH metabolites in birds resulting from chronic environmental exposure, i.e. non-oil spill exposure. Direct measurement of PAH metabolites is sensitive and tracks PAH doses better than bioassays (van Schanke et al. 2001). Additionally, even if the bioassay was positive, there would not be a way to definitively attribute the response to PAHs, since bioassays are non-specific. Therefore, measurement of a panel of PAH metabolites is an ideal method for estimating cormorant PAH exposure across differentially contaminated sites.

Thesis objectives

Few studies have examined microsatellite mutations in genetically variable natural populations exposed to ambient environmental pollution. This aspect is important, since one goal may be to continue this line of research with other wildlife species and humans, which are also genetically variable populations. This research adds another similarly exposed species to the small number of species that have been studied for toxic effects in Hamilton Harbour.

The primary objective of my thesis was to test whether families of cormorants nearest to the industrial core site in the Harbour would have higher mutation rates using a pedigree screening approach. Our hypothesis was that airborne contamination from Hamilton Harbour industries induces microsatellite mutations in exposed wild cormorants. We predicted a gradient in mutations, with highest rate in the site closest to industrial sources of PAHs, and lowest numbers from the cleaner reference site on Lake Erie. My second objective was to quantify PAH metabolites in tissues and samples from cormorants, to test the hypothesis that proximity to a more contaminated site is correlated with higher concentrations of PAH metabolites in a natural population of cormorants. Together, these studies allow me to test the overall hypothesis that sites where cormorants have higher PAH metabolites are the sites with higher rates of microsatellite mutations, implying exposure to PAHs as a cause of these mutations. Thirdly, I wanted to assess whether cormorants at the three sites have different diets, to see if feeding on different species had the potential to promote variation in contaminant uptake. I predicted that the two pollution-exposed colonies in Hamilton Harbour would have similar diets, such that if this was true the differences in exposure might be more likely to result from airborne exposure. Therefore, if increased mutation rates were found at Pier 27 (the site closer to major PAH releases) as compared to Farr Island (the site in Hamilton Harbour farther from PAH releases), this would imply higher exposure to industrial PAHs as the main cause of mutations.

Table 1. Selected Polycyclic Aromatic Hydrocarbons (PAHs) released into ambient air at Hamilton Harbour, Ontario, Canada by the two integrated steel production complexes, the main producers of PAHs in the region (two other industries reported extremely small amounts and are not included). Includes PAHs 'Measured by HAMN': One of the eight PAHs measured by the Hamilton Air Monitoring Network high volume air samplers and 'US EPA Priority Pollutant': Designated as one of 16 original 'Priority Pollutants' in the PAH category by the Environmental Protection Agency of the United States. IARC classifications are as follows: Group 1: carcinogenic to humans; Group 2A: probably carcinogenic to humans; Group 2B: possibly carcinogenic to humans; Group 3: not classifiable as to its carcinogenicity to humans. *Naphthalene, having only two rings, is a bicyclic aromatic hydrocarbon and not usually considered a true PAH. Data from HAMN (Hamilton Air Monitoring Network), NPRI (National Pollutant Release Inventory 2009 and 2010).

PAH (IUPAC name)	Number of rings	IARC Group	Hamilton Harbour Air Releases 2009 and 2010	Notes
7H-Dibenzo[c,g]carbazole	5	Group 2B	0.45 kg	
Acenaphthene	3	Group 3	1067.37 kg	US EPA Priority Pollutant
Acenaphthylene	3	not available	436.3 kg	US EPA Priority Pollutant
Anthracene	3	Group 3	0.8 kg	US EPA Priority Pollutant
Benzo[a]anthracene	4	Group 2B	512 kg	Measured by HAMN; US EPA Priority Pollutant
Benzo[a]pyrene	5	Group 1	489 kg	Measured by HAMN; US EPA Priority Pollutant
Benzo[b]fluoranthene	5	Group 2B	483 kg	Measured by HAMN; US EPA Priority Pollutant
Benzo[g,h,i]perylene	6	Group 3	29.3 kg	Measured by HAMN; US EPA Priority Pollutant
Benzo[j]fluoranthene	5	Group 2B	39.2 kg	
Benzo[k]fluoranthene	5	Group 2B	311 kg	Measured by HAMN; US EPA Priority Pollutant
Chrysene	4	Group 3	not reported (but see Chap. 2, Fig. 4)	Measured by HAMN; US EPA Priority Pollutant

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Fluorene	3	Group 3	42.51 kg	US EPA Priority Pollutant
Fluoranthene	4	Group 3	1082 kg	US EPA Priority Pollutant
Dibenzo[a,i]pyrene	6	Group 2B	481 kg	
Dibenzo[a,h]anthracene	5	Group 2A	537.7 kg	Measured by HAMN; US EPA Priority Pollutant
Dibenz[a,j]acridine	5	Group 2B	0.46 kg	
Indeno[1,2,3-c,d]pyrene	6	Group 2B	187.7 kg	Measured by HAMN; US EPA Priority Pollutant
Naphthalene*	2*	Group 2B	28.4 tonnes	US EPA Priority Pollutant
Phenanthrene	3	Group 3	1887 kg	US EPA Priority Pollutant
Pyrene	4	Group 3	885 kg	US EPA Priority Pollutant

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Chapter Two: Microsatellite DNA mutations and benzo[a]pyrene metabolites in Double-crested Cormorants (*Phalacrocorax auritus*) associated with exposure to PAH-containing industrial air pollution in Ontario, Canada

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Abstract

Hamilton Harbour, Ontario, Canada is one of the most polluted sites on the Great Lakes and affected by airborne and sedimentary contamination as a result of both heavy vehicle traffic and industrial steel emissions. Mutagenic Polycyclic Aromatic Hydrocarbons (PAHs) are present at very high concentrations in the air and sediment of Hamilton Harbour. We used five highly variable DNA microsatellites to screen for mutations in 97 families of Double-crested Cormorants (*Phalacrocorax auritus*) from three wild colonies, two in Hamilton Harbour and one in cleaner northeastern Lake Erie. Mutations were identified in all five

microsatellites at low frequencies, with the majority of mutations found in chicks from the Hamilton Harbour site closest to industrial sources of PAH contamination. A Phase I metabolite of the PAH benzo[a]pyrene identified by LC-MS/MS in bile and liver from Hamilton Harbour cormorant chicks suggest that these cormorants are exposed to, and metabolizing, PAHs, highlighting their potential to have caused the observed mutations.

Introduction

Polycyclic Aromatic Hydrocarbon (PAH) contamination is a serious concern in highly contaminated Hamilton Harbour, Ontario, Canada, home to both heavy industry and major highways. PAHs are ubiquitous byproducts of incomplete combustion of organic materials and are found in air pollution resulting from both integrated steel production and vehicle exhaust; both affect Hamilton Harbour due to thousands of kilograms of industrial PAH releases (NPRI 2009, 2010) and nearby heavy vehicle traffic. PAHs and their metabolites can cause endocrine disruption, alterations in physiology and gene expression, DNA damage, and cancer (Belpomme et al. 2007). In environmental settings, PAHs are often found in complexes such as NPAHs (nitrogenated PAHs), increasing their mutagenicity (Wang et al. 2011), (Layshock 2010). PAHs are metabolized in vertebrates to form reactive diol intermediates, which form adducts with nucleotides in DNA, causing mutations (Albers 2006). Previous research in Hamilton Harbour demonstrated higher minisatellite germline DNA mutation rates relative to cleaner reference groups in herring gulls (*Larus argentatus*; Yauk & Quinn 1996; Yauk et al. 2000) and higher ESTR (expanded simple tandem repeat) mutations in mice (*Mus musculus*; Somers et al. 2002, 2004), with particulate matter containing PAHs as a suspected

cause. Mice breathing only filtered air in sites exposed to ambient air pollution showed decreased mutations, suggesting that breathable particulate is responsible for mutation induction (Somers et al. 2004).

Miller (2009) identified microsatellite mutations in fathead minnows (*Pimephales promelas*) exposed *ex situ* to sediment from Hamilton Harbour's highly PAH-contaminated Randle Reef. Anmarkrud et al. (2011) compared mutation rates in highly variable microsatellite markers in tree swallows (*Tachycineta bicolor*) from Windermere basin, Hamilton Harbour to those from a relatively unpolluted area of Eastern Ontario, but neither study found statistically significant differences between reference and exposed groups. While Ellegren et al. (1997) reported elevated microsatellite mutations in tree swallows breeding near Chernobyl compared to those from a less contaminated site, a significant increase in microsatellite mutations as a result of non-radioactive ambient environmental contamination has not been reported to date.

Double-crested Cormorants (*Phalacrocorax auritus*) are large-bodied migratory waterbirds that breed in colonies on the Great Lakes; due to their size and abundance, they are ideal for toxicological studies. If cormorants are being exposed to PAHs at contaminated Hamilton Harbour sites as expected, PAH metabolites should be present in their body tissues. PAHs are actively biotransformed (metabolized) in most vertebrate animals, first forming Phase I metabolites (hydroxylated or hydroxy-PAHs). Measuring PAH metabolites in tissues (as opposed to using bioassays that assess only the toxicity of PAH exposure) provides a sensitive PAH exposure marker which can track dose well (van Schanke et al. 2001). In oiled common guillemots (*Uria aalge*), the majority of PAH compounds were found as hydroxylated

metabolites (2-7 times more metabolites than parent PAHs, Troisi et al. (2006). To our knowledge no study has yet shown PAH metabolites in birds resulting from chronic ambient environmental exposure, i.e. non-oil spill exposure. We hypothesized that due to the high airborne PAH concentrations in Hamilton Harbour, we would detect various metabolites in cormorant tissues.

To evaluate the effect of pollution exposure, we chose three cormorants colonies at varying distance to sources of PAH-containing emissions. Within Hamilton Harbour, we sampled at one colony close to coking ovens used in industrial steel production, where prevailing winds blow pollution to the colony, and another across Hamilton Harbour which is both farther away and less often downwind of steel emissions. The highest exposure was at the site next to the industrial core, and the lowest in our reference site at Lake Erie. By using three different sites with varying exposure to PAHs, an examination of the impact of PAHs on mutation rates could be possible, especially if diet is the same (held constant) at the two Hamilton Harbour sites. We predicted that the mutation rate will increase with decreasing distance to the Hamilton Harbour industrial emission sources, such that the highest mutation rate will be found at the site closest to the steel mills and the lowest in our reference colony on Lake Erie. Here we report germline microsatellite mutation rates in families of Double-crested Cormorants from two colonies in Hamilton Harbour and one colony on Lake Erie, demonstrating that microsatellite mutation induction is correlated with increased exposure to airborne industrial pollution.

Methods

Study sites and sampling procedures

Three similarly sized (each containing approximately 1000 breeding pairs) colonies of ground-nesting cormorants on the lower Great Lakes, Ontario, Canada with varying degrees of sedimentary and airborne contamination were chosen for sampling.

The study colony at Pier 27, Hamilton Harbour, western Lake Ontario (43° 17' N, 79° 49' W; Figure 1) was the closest to a major source of industrial pollution, approximately two kilometres east of two large integrated steel mills, and 350 metres west of a 6-7 lane highway (the Queen Elizabeth Way, QEW) as well as Eastport Drive. Prevailing winds in the Harbour originate from the southwest, (Wallace et al. 2009, 2010), placing Pier 27 downwind of industrial releases on a majority of days during the cormorant breeding season. Farr Island, Hamilton Harbour, was slightly farther away (approximately four kilometres; Figure 1) from the industrial core, but also directly west of the same major highway (the QEW). Farr Island was converted to underwater fish habitat in 2010. For further descriptions of these two Hamilton Harbour sites see Somers et al. (2007) and (Gebauer et al. 1992); for another detailed map including these two sites see Figure 1 in Quinn et al. (1996). For our cleaner reference site, we chose a rural non-industrialized area in northeastern Lake Erie on the basis of this region having very low levels of contamination relative to Hamilton Harbour (McCarthy et al. 2004) plus some of the least contaminated eggs in a Great Lakes-wide herring gull (*Larus argentatus*) study (Weseloh et al. 2006), suggesting that impacts of anthropogenic contamination would be minimal. Mohawk Island (42° 50' 2.5008" N, 79° 31' 22.497" W) is a National Wildlife Area in northeastern Lake Erie, near Rock Point and the mouth of the Grand

River, approximately two kilometres from shore (King & de Solla (2010)).

We trapped adult cormorants on their ground nests in these three colonies during peak breeding season between May and July in both 2009 and 2010. We used approximately 1 x 1.5 x 0.5 metre box traps made with chicken wire over a steel rod frame and a two-piece hinged wooden trigger connected to the back of the trap with clear monofilament line. We chose nests with mainly or all non-mobile chicks and avoided nests which still contained eggs. We took whole blood samples (up to 18 mL) by brachial venipuncture from adult cormorants using 23 or 25 gauge Sarstedt Multifly butterfly catheter needles connected via Luer-Lock to either 6 or 12 mL Norm-ject syringes. Once two presumed parents had been blood sampled at one nest, we took blood samples from all of the chicks present in that nest. For small chicks (less than 600-800 grams) we used a 27 gauge 1/2 inch (grey) Becton-Dickinson PrecisionGlide needle for brachial venipuncture followed by blood collection with one heparinized glass capillary tube. For older juveniles that we judged to be large enough, we used a 25 gauge 5/8 inch (blue) Becton-Dickinson PrecisionGlide needle connected to a 1 mL plastic syringe. In total over the two field seasons, we blood sampled 113 complete cormorant families (47 from Pier 27, 36 from Farr Island, and 30 from Mohawk). All needles for blood sampling were rinsed in 0.5 M EDTA immediately before use to prevent blood coagulation inside the needle. Blood for genetic analysis was deposited directly into 1 mL pH 8.0 Queen's lysis buffer (Seutin et al. 1991). In 2010, we collected fecal samples from both chicks and adults whenever possible, by holding 1.5 mL plastic tubes close to the cloaca. We also opportunistically collected 18 dead chicks that appeared to have died of heat or cold stress from all three colonies during the course of field work in 2010. Sampling and collection

was approved by Environment Canada's Animal Care Committee and McMaster University's Animal Research Ethics Board (AUP # 08-06-31), samples collected under an Ontario Ministry of Natural Resources (OMNR) Wildlife Scientific Collector's Authorization (Number 1051384), and cormorants banded under a Scientific Permit to Capture and Band Migratory Birds from the Canadian Wildlife Service (permit number 10529 AB).

Microsatellite Genotyping

DNA was amplified and sized to allow for allele comparisons between parents and offspring. DNA from sampled cormorant families was extracted from lysed blood samples using an ammonium acetate salt extraction method (Sambrook & Russell 2001). We selected two microsatellites (CORM 4A and CORM 5A2) from a set isolated from cormorant genomic DNA (T.W. Quinn and M. KC unpublished), using a rapid capture method (St. John & Quinn 2008) (Table 1). We selected three microsatellite markers (COR 01, COR 03, and COR 06) with high heterozygosity and number of alleles from the 24 in Fike et al. (2009), for a total of five microsatellite loci used (Table 1). We chose 'perfect' microsatellites (where repeats are not interrupted by other intervening nucleotides) where possible, resulting in 3 perfect and 2 imperfect microsatellites. Microsatellite genotyping errors are likely in most studies and can cause research conclusions to be seriously flawed (Hoffman & Amos 2005). Therefore, for quality control purposes, we selected only microsatellites with tetranucleotide repeats, which can be easier to score (Guichoux et al. 2011) compared to dinucleotide repeats. The longer the repeat unit, the less chance for common issues such as adenylation of the PCR product or slightly different migration during capillary electrophoresis to impact scoring results (Macdonald et al. 2011) because even single unit mutations would be at least 4 bp away from

the original allele. Radioactive (^{33}P) PCR products were first electrophoresed on 6% denaturing polyacrylamide gels for visual inspections of band stutter patterns and allele size ranges. Primers were then ordered fluorescently labelled with HEX (green), 6-FAM (blue, IDT DNA, Coralville, IA) and NED (yellow, Applied Biosystems, Inc.) such that when similarly sized PCR products overlapped, differently coloured dyes would still enable identification. Forward primers were labelled, except when proximal G base quenching would have caused interference with the dye (Behlke et al. 2005); in these cases (primer sets COR 06 and CORM 5A2) the reverse primer was labelled instead (Table 1). Multiplex PCR was then performed in 10 μL volumes with 0.15-0.2 μM each forward and reverse primers, 1x +KCL -MgCl₂ buffer, 1.5 mM MgCl₂, 1 U Taq polymerase (all Fermentas Life Sciences), bovine serum albumin (BSA, Sigma), and 0.2 mM each dNTPs (GeneDirex). The cycling program began with an initial denature at 94.6°C for two minutes, then 30 cycles of denaturation at 94°C for 30 seconds, annealing at 55.8°C for 15 seconds, extension at 72°C for 15 seconds, then a 10 minute extension at 72°C followed by a 45 minute extension at 60°C.

Loci that would not amplify properly in a multiplex system were amplified separately and subsequently pooled with other microsatellite PCR products, then analyzed together in the same well (sometimes referred to as pseudo-multiplexing or poolplexing, (Guichoux et al. 2011). Amplifications were verified on 1% agarose gels before genotyping, then precipitated with ethanol and ammonium acetate in 96-well plastic plates to removing excess primer, magnesium chloride, and dNTPs before genotyping. As little as 4 μL and up to 15 μL of PCR product was ethanol precipitated with cold 95% ethanol and 7.5 M ammonium acetate, followed by 35 minutes of centrifugation at 2161 g (4100 rpm) and a wash step using 150 μL

of 70% ethanol. Pellets were usually resuspended in the original volume of water, except in cases when concentrating the DNA was deemed necessary (judging by a weak band on the agarose gel). In these cases they were resuspended in a volume of water smaller than their original PCR product volume.

PCR products were genotyped by capillary electrophoresis on an ABI 3730 DNA Analyzer at the Natural Resources DNA Profiling and Forensic Centre, Trent University, Peterborough, Ontario using an ET550-R size standard. Electropherograms were transferred as .fsa files and alleles sized without knowledge of site nor age (i.e. adult versus chick) by one observer (LEK) using GeneMarker V 1.91 (SoftGenetics LLC.), using spike removal, pull-up correction, and enhanced baseline subtraction on the raw data trace and the recommended Local Southern allele sizing method. The automated allele calling available in many programs can be a major source of genotyping error (Dewoody et al. 2006) so individual sizing of each peak on the electropherogram was used instead to avoid this problem. One primer (forward COR 01, Table 1) was used with two different dyes, but after careful examination no dye shift was apparent (Sutton et al. 2011). Allele sizes were determined by examining frequency distributions of raw, unbinned lengths in base pairs including decimals, i.e. true allele sizes (Guichoux et al. 2011). In cases where allele peaks were small or unclear, PCR and genotyping was repeated until they were clear enough for unambiguous scoring (two to three times).

As recommended by Dewoody et al. (2006) we also followed a number of multiple tube approach procedures to produce the best quality genotyping possible. We amplified and subsequently genotyped a small percentage of samples in duplicate and triplicate. We also

separately extracted DNA twice from single samples then separately amplified, as well as taking two blood samples from the same bird, then extracting DNA and amplifying each sample separately, to ensure that microsatellite genotypes remained the same under all of these conditions. Lastly, of the families with identified mutations, we repeated PCR and genotyping for the chick(s) in question (4/8 families, or 50% of cases) or chick(s) plus parents (3/8 families, or 37.5% of cases), such that 7/8 cases (87.5%) were reamplified and regenotyped to some degree.

Adult cormorants were sexed following Fridolfsson & Ellegren (1999), using the touchdown PCR procedure outlined, modified by the addition of bovine serum albumin (BSA), and PCR products were visualized on 1% agarose gels. To increase our confidence that captured families represented the two actual parents of the chick, we sexed both parents in a subset (17.5 %) of families to demonstrate that each pair in fact represented one male and one female (these chosen families had at least one parent requiring sexing for our concurrent diet study, Chapter 3)

Differentiating extrapair events, mistrapping and mutations

After extrapair parentage or incorrect trapping of a parent was ruled out, alleles seen in the offspring's genotype which were different sizes than those in either parent's sample (at least one repeat unit or 4 base pairs different) were scored as mutations. However, since cormorants have high site fidelity (Scherr et al. 2010), it is possible that relatives breed side by side in the same colonies. Therefore, extrapair paternity or maternity could be difficult to differentiate from true mutations, but the use of multiple variable microsatellites helps to alleviate this problem. The higher the number of microsatellites which do not match, the

higher the likelihood of this event being an extrapair event. We used a probability-based measure (cumulative probability of resemblance, P_{RaCum} , Ibaruchi et al. (2004) that takes this into account, using allele frequencies at alleles shared between the chick and the putative parent. We found allele frequencies (for each site separately) and checked for null alleles in Cervus 3.0.3 (Field Genetics Ltd.; Marshall et al. 1998; Kalinowski et al. 2007). We then calculated P_{RaCum} for chicks with any non-matching alleles using Equation 1 then Equation 3 of Ibaruchi et al. (2004), which represents the probability of having randomly sampled an adult with that matching genotype. When P_{RaCum} was less than 0.001, we retained the chick in the analysis; when it was higher we removed it. This represents a less than 0.1% chance that this incorrect parent, sharing these exact alleles, was randomly sampled in the colony, so the probability that this is the correct parent is thus at least 99.9%. We chose this value as it is lower than others previously used in the literature (e.g. >0.005 , Evans et al. (2008)), and a conservative approach is appropriate for the identification of induced mutations, especially in a species with high natal philopatry. Additionally, this probability would correspond to approximately less than 1 in 1000 individuals, a biologically relevant cutoff since each colony contained approximately 1000 adult cormorants of each sex. Therefore, our cutoff is so low that it would represent no adult in the colony available to be the incorrectly trapped adult parent. This value is also the same as the very strict cutoff used for human microsatellite confirmation of parentage (Jochens et al. 2011; Kayser et al. 2000). In families with only one chick where this chick had a P_{RaCum} high enough that it had to be removed from analysis, or where all chicks had a high P_{RaCum} this meant the removal of the entire family from the analysis ('removed families', $n = 16$). However, in multi-chick families, where the genotype of one chick did not match those of siblings nor those of parents, we interpreted these mismatched

genotypes as consistent with some type of extrapair event or adoption, since extrapair offspring will be contained within the same clutch as the within-pair offspring. In these cases we removed the one mismatched chick from analysis ($n = 8$ chicks, 'removed chicks') but kept the remainder of chicks and therefore the family in the analysis.

The cumulative probability of resemblance is only designed for cases where the chick in question matches one parent (usually the mother) at all loci, but mismatches the other at one or more loci (Ibarguchi et al. 2004). In cases where neither parent matched the chick(s), we removed the chick(s) from analysis without the ability to calculate P_{RaCum} . In some of the families, there are almost complete mismatches between the parental microsatellite genotype and that(those) of the chick(s), consistent with an obvious problem such as incorrect trapping of one or both parents. In these cases we removed the chick without calculating P_{RaCum} since if there were no alleles shared we could not calculate this probability. Overall, 16 families were removed from analysis out of the original 113 families trapped, representing 14.1% of the original families trapped. Of these removed families, 8 were from Pier 27, 6 from Farr Island, and 2 were from Mohawk Island. This demonstrates that even if there were a bias towards excluding some families from analysis with respect to site, the bias would have been in a conservative direction since a higher number and fraction of families were removed from Pier 27 and Farr Island (approximately 20% of the original families were excluded from each of Pier 27 and Farr Island, while only 7% of original families were excluded from Mohawk Island). These removed families showed mismatched genotypes with one parent, consistent with a incorrect trapping of one of the parents. This left 97 families for statistical analysis (39 from Pier 27, 30 from Farr Island, 28 from Mohawk Island). Each chick ($n = 248$, 83 from Pier

27, 92 from Farr Island, and 73 from Mohawk Island) in these 97 families had a total of 10 alleles scored (5 microsatellite loci with two alleles per locus), amounting to a total of 496 alleles scored per locus and a total of 2480 alleles scored in chicks, representing 2480 meioses. If an allele was present in a chick which was not seen in either of the parents, but either of the parents was homozygous at that locus, this was not scored as a mutation since this could have represented allele dropout in one or both of the parents. Alleles were scored as being different from the parental alleles if they were scored as being in the next allele bin, such that 1 and 2 bp differences from parental alleles were not scored as mutations. After mutations were identified in the five loci, the numbers of perfect and imperfect loci used in the study were compared to the numbers of mutations identified in each type of locus with a 2 x 2 contingency table chi square analysis to test whether there was a bias towards identification of mutations in either type of loci.

Comparing mutations among sites

Mutations within families are often compared by using generalized estimating equations (GEE), e.g. Tsyusko et al. (2007), Miller (2009), which can be a good choice for data containing observations (here, mutations) within clusters (here, families) that may be correlated due to relatedness (Hanley 2003, Halekoh et al. 2006). Specifically, multiple mutations within a family may be correlated because a certain individual parent may be more likely to pass on mutations. Therefore, as an example, two chicks both with mutations in a nest of two does not equate to two samples, nor one sample, but a calculated “effective sample size” (Hanley 2003) between 1 and 2. However, the GEE model did not fit these data properly because of the relatively low overall number of mutations that were identified (Ben

Bolker, pers. comm.). GEE models, like the generalized linear models they are based on, give biased results when using small sample sizes (Paik 1988, Morel et al. 2003), and regardless of the number of families, chicks, or loci used here, the number of mutations is the sample size which was too small for GEE. Standard contingency table analysis would force the treatment of every family as simply a binary yes/no in terms of whether any mutations were identified anywhere in any chicks in the family, causing a moderate loss of power.

Furthermore, they would have to be analyzed by simulation in any case because the mean numbers of mutations per family are far below 5 (which violates the basic assumptions for standard contingency table analyses). Therefore, mutation rates were instead compared among sites using a permutation test implemented in R (R Development Core Team 2011).

Permutation tests are conceptually simple and have a variety of advantages including being non-parametric and relying on few assumptions about the data (May & Hunter 1993). Our null hypothesis was that the probability of mutations was identical at all three sites. We calculated a test statistic for departures from the null hypothesis by weighting mutations occurring at Mohawk by 0, at Farr Island by 0.5, and at Pier 27 by 1.0, in accordance with our alternative hypothesis that there would be a gradient in probability of mutations by site, with Mohawk having the lowest number of mutations, Pier 27 having the highest, and Farr Island intermediate, with a number of mutations somewhere between those other two sites. When more than one chick in the same family showed the same mutation at the same locus, this was counted as only one mutation; this conservative approach assumes the mutations in both chicks result from the same mutation event during gametogenesis. To compute the null distribution of the test statistic, we created 2000 permutations of the data, scrambling the families with respect to site in a way that preserved the total number of mutations and the

total number of samples per site, and computed the value of the test statistic for each one. We then computed the p-value for the null hypothesis by computing the fraction of the null-distribution outcomes greater than or equal to the test statistic computed for the observed data. We did not compute further post-hoc tests (pairwise comparisons) to identify where the difference lay among the three sites, since we had already shown the overall pattern was significant. The issue of the use of multiple comparisons is currently contentious, and the *Journal of Probability and Statistics* (2011) has reported that “some practitioners...have even advocated ending the use of multiple comparison methods”. Multiple pairwise comparisons could confuse the issue further since using a correction could eliminate significance, especially in cases such as this which are on the edge of statistical significance (Bolker 2008). Increasing the number of tests used can then end up being confusing and complicating interpretation (B. Bolker, J. Dushoff, pers. comm.). Other authors have also advocated the view that pairwise comparisons should be avoided (e.g. Smith 2004).

PAH metabolite analysis

For liquid chromatography-tandem mass spectrometry (LC-MS/MS), we chose six hydroxylated metabolites as indicators of contamination to evaluate PAH exposure in cormorants in the three colonies: metabolites of five PAHs, 1-hydroxypyrene, 2-hydroxyphenanthrene (Dr. Ehrenstorfer, Augsburg, Germany), 1- and 3-hydroxybenzo[a]pyrene, benzo[a]pyrene-7,8-diol (Midwest Research Institute, Kansas City, Missouri), and one metabolite of the bicyclic aromatic hydrocarbon naphthalene, 2-hydroxynaphthalene (Toronto Research Chemicals, Toronto, Canada).

Fecal samples from both adults and chicks (n = 19, up to 0.7 g wet weight) were enzyme digested overnight by incubating with 20 μ L of *Helix pomatia* enzyme with beta glucuronidase and aryl sulfatase activity (Sigma-Aldrich) and 1 mL of buffer solution (sodium acetate, pH 5); samples were subsequently air dried to obtain a dry weight (up to 1.04g). Bile samples (n = 18, up to 0.3 g wet weight) were removed from the gallbladder with a needle and liver samples (n = 8, up to 0.5g wet weight) dissected out of thawed cormorant chicks, then frozen at -15°C until enzyme digestion. Bile and liver samples were subjected to an additional hydrochloric acid hydrolysis step (van Schanke et al. 2001) to hydrolyze possible Phase 2 metabolites (sulfate and glucuronate conjugated PAHs), then neutralized with sodium hydroxide prior to extraction. 100 μ L of Bisphenol A in methanol (for a final concentration of 100 ng/mL) was added to all samples as an internal standard, then samples were extracted twice with 5 mL 100% ethyl acetate, centrifuged for 10 minutes at 3000 rpm, then evaporated to near dryness under a stream of nitrogen and reconstituted in 1 mL of 30:70 methanol:water to match the initial composition of the mobile phase. All glassware was washed with Contrad 70 and then rinsed in acetone/pentane prior to use to remove any interfering contaminants. Extraction blanks were processed alongside each set of samples through the same method to check for any sources of contamination resulting from glassware, equipment, or buffers.

Samples were analysed by LC-MS/MS using an Agilent 1100 LC system coupled to an API 2000 system (Applied Biosystems MDS Sciex Q trap), operated in APCI (atmospheric-pressure chemical ionization) mode. The Agilent 1100 LC was modified by removing the mixing chamber to allow faster gradients. Sample solution (25-75 μ L) was injected onto an

Xterra C18 guard column (50 x 2.1 mm, 3.5 µm particle size) with a Phenomenex Gemini analytical column (50 x 2.1 mm, 3µm particle size). The mobile phase consisted of Milli-Q water (A phase) and methanol (B phase), with 0.06% ammonium hydroxide (Fisher Scientific) in each. The gradient was held at 30:70 methanol:water for the first minute of the run and then was increased linearly to 90% methanol over 3 minutes and held at 90% methanol for 3 minutes before returning to the initial conditions. After injection, the sample was trapped onto the guard column and the eluent was diverted to waste, by means of a switching valve. After three minutes, the valve was switched and the flow was directed into the analytical column and the ion source. Nitrogen was used for the curtain, heater, collision, and nebulizing gas. The probe temperature was set at 450°C, with a nebulizer current of -2 mA (milliamperes). Data was collected and peaks integrated using Analyst 1.5.2 software (Applied Biosystems/MDS).

Wind directions and airborne PAH concentrations

The earliest cormorants in these colonies return from wintering in late March and early April ('Migration', Fig. 7, Hatch & Weseloh 1999); relevant breeding period exposure to genotoxins in Hamilton Harbour could have begun at that time, and continued until mid-June. Therefore, we defined the 'potential exposure period' of the adult breeding cormorants in these colonies to be March 20 to June 14. Wind direction often changes hourly, causing Hamilton Harbour cormorant colonies to be alternately downwind or upwind of industrial emissions. In terms of exposure, and therefore possibly mutation induction, the amount of time spent downwind or upwind can be critical ((Sofowote et al. 2010b)(a)). Direct wind from the nearby heavy traffic may also be important. Accordingly, we collected wind direction and

airborne PAH concentrations for this period in both study years of 2009 and 2010 from the Hamilton Air Monitoring Network (HAMN: hamnair.ca) and the Ontario Ministry of the Environment (MOE). Hourly average wind direction at 10 metres height from the ground at Station 29167 (Figure 1) for every day during the potential exposure period was downloaded from hamnair.ca. This station is the closest to the cormorant colony at Pier 27, approximately 600 metres southwest, which causes it to be downwind of the industrial area during prevailing southwesterly winds.

Concentrations of airborne PAHs from Station 29547 of the Hamilton Air Monitoring Network were provided by an Air Quality Analyst of the Ontario Ministry of Environment (MOE); this is the closest site sampling PAHs, located at Pier 25 approximately 1.2 kilometres southeast of the Pier 27 cormorant colony (Figure 1). Modified high volume air samplers with Teflon filters sampled air for 24 hour periods for 8 PAHs for GC/MS analysis (benzo[a]anthracene, benzo[a]pyrene, benzo[b]fluoranthene, benzo[g,h,i]perylene, benzo[k]fluoranthene, chrysene, dibenzo[a,h]anthracene, and indeno [1,2,3,cd] pyrene). Air samplers recorded PAHs every 12 days, leading to eight sampling days during the potential exposure period of each year, for a total of 16 PAH sampling days in both years. Adding the 8 PAH measurements from each day, we calculated a total PAH measurement for each day of sampling. We then compared the total PAH concentrations in each study year (2009 and 2010) to those from the same station in other years reported in Somers et al. (2004) and Yauk et al. (2008) with a mixed linear model to determine if there were differences in PAHs in Hamilton Harbour during these studies. We also downloaded PAH emission data from industries in the Hamilton Harbour region for 2009 and 2010 from Environment Canada's

National Pollutant Release Inventory (ec.gc.ca/inrp-npri).

Combining measures of hours downwind and exposure days, we followed the previous attempts by others (Somers et al. 2004, Yauk et al. 2008; Anmarkrud et al. 2011) to quantify the percentage of days exposed cormorants at both Hamilton Harbour colonies were subject to winds blowing from the industrial area. We grouped the exposure days into the categories originally used by Somers et al. (2004). While Anmarkrud et al. (2011) used 'days of field work' to assess exposure, we counted days in the breeding period, as this was when breeding cormorant gametes would actually be exposed to air pollutants and is therefore a more accurate measure. We measured wind directions which cause Pier 27 to be downwind of the industrial area in two different ways, westerly wind (wind originating from 180° to 360°, a span of 180°, which caused Pier 27 to be approximately downwind) and southwesterly wind, wind originating from 180° to 270°, a span of 90°, causing Pier 27 to be directly downwind). We estimated that southerly wind would place Farr Island approximately downwind of the industrial area, so we calculated hours of southerly wind (wind originating from 90° to 270°, a span of 180°).

Results

Mutation rates

We identified 8 cormorant families in total containing chicks with at least one mutation out of 97 families (8.2% of families contained at least one chick with at least one mutated allele, Tables 2 and 3). This represented 11 chicks with any mutations out of 248 chicks

screened or out of 2480 meioses scored (4% of chicks had at least one mutation). We identified at least one mutated allele in all five microsatellite loci screened. In 2 nests, there were 2 chicks in the family that each had the same size mutated allele not seen in the parents, so we counted these two cases as just one unique mutation each since they may result from the same cell division event. Two chicks in the same family each had mutations in 2 different loci, which we counted as two mutations since they result from different cell division events. Overall there were a minimum of 11 unique mutations, 8 of which were at Pier 27, 2 at Farr Island, and 1 at Mohawk Island. This represents 6 families at Pier 27, 1 family at Farr Island, and 1 family at Mohawk Island with any mutations (at least one mutation). The mutation rate at Pier 27 was 4.4 times higher than that at Farr Island, and 7 times higher than that at Mohawk Island, and mutation rate varied significantly among the three colonies (permutation test statistic = 9, $p = 0.041$).

The numbers of mutations identified varied among loci (Tables 2 and 3), with a low of 1 mutation identified in COR 01 to a high of 3 unique mutations identified in each of COR 03 and COR 06 (each had 4 mutations total). Per-allele mutation rates for the 5 loci including all three sites ranged from 0.002 to 0.006 (Table 3), giving an overall per-allele mutation rate of 0.0044 including all five loci. Of the 11 unique mutations, 3 (27%) occurred in imperfect microsatellite loci (i.e. COR 01 and CORM 5A2), while 8 (73%) were found in perfect loci (i.e. COR 03, COR 06, and CORM 4A). There was no significant bias related to the frequency of mutations identified in each type (perfect versus imperfect) of microsatellite locus ($\chi^2 = 0.259$, $\alpha = 0.05$, $df = 1$, $n = 16$). In 2 cases (18%) of the 11 unique mutations we could not identify which allele was the putative progenitor allele since each parent had the same genotype or

alleles at that locus. In the remaining cases, if we assumed the source of the chick mutant allele was the parental allele closest in size, 3/9 mutations, or 33%, arose from the male parent. Using that assumption, 5/11 (45%) of mutations consisted of gains in repeat unit(s) and 6/11 (54%) represented a loss of repeat unit(s), and 5/11 (45%) mutations demonstrated multi-repeat-unit changes while 6/11 (54%) demonstrated a change of 1 repeat unit. Null alleles were not suspected in any of the five loci ($F(\text{Null}) = -0.01$ to -0.04 , deviations from Hardy-Weinberg equilibrium were not significant). In the 11 unique mutations, we never saw more than 2 alleles at the mutating locus in question.

Of the 8 chicks excluded from mutation analysis from families that remained in the analysis, 6 chicks mismatched both parents at one or more loci (some chicks mismatched both parents at up to 3 loci), while 2 chicks mismatched the putative male parent at 3/5 and 4/4 loci but matched the female parent. Of the 8 excluded chicks, 7 were from Pier 27 and one was from Mohawk Island. In the 17 families in which we sexed both parents, all consisted of one female and one male.

Wind and airborne PAH pollution in Hamilton Harbour

The National Pollutant Release Inventory (NPRI 2009, 2010) showed Hamilton Harbour area industrial PAH emissions in the range of thousands of kilograms of PAHs per year during the two years of field work for this study (see Chapter One, Table 1). We characterized the potential exposure of airborne PAHs to cormorants in Hamilton Harbour during the breeding period (March 20 to June 14), by quantifying the wind direction from the major source of PAHs (steel mills) relative to the colonies. Pier 27 was often downwind of the

industrial core: of the 87 days during the potential exposure period in 2009, 53% of days received at least 10 hours of westerly wind (Table 5). During the potential exposure period of 87 days in 2010 the wind blew from the west for at least 10 hours per day on 53% of days as well, although in 2010 a lower proportion of days with 24 hours of westerly wind were recorded (24% vs 15%). Farr Island was less often downwind: of the 87 days in 2009, 32% of days had at least 10 hours of wind, and in 2010 37% of days had at least 10 hours of wind. Only 8 and 4% of days at Farr Island had more than 19 hours of wind, compared to 26 and 30% at Pier 27 (in 2009 and 2010 respectively). The proportions of hours that Pier 27 was downwind of the industrial core did not differ between 2009 and 2010; furthermore the amount of time Pier 27 spent downwind in 2009 and 2010 were similar when compared to earlier studies near Pier 27 ($F = 0.26$, $df = 5$, $p = 0.92$; 2000: Somers et al. 2004, 2004: Yauk et al. 2008). Airborne PAH concentrations (total of 8 PAHs) at Station 29547, Pier 25 (near the Pier 27 cormorant colony; Figure 1), ranged between approximately 2-15 ng/m³ during *in situ* exposure studies using caged mice near Pier 27 from May 21 to July 20, 2004 (Yauk et al. (2008), and from the current study, ranged between approximately 1-25 ng/m³ in 2009 and 1-58 ng/m³ in 2010. PAH concentrations at Pier 25 (total of 26 measured PAHs) ranged between approximately 2-28 ng/m³ during the Somers et al. (2004) mouse exposure study (2000). Mean PAH concentrations at Pier 25 in these four years were not different ($F = 0.16$, $df = 27$, $p = 0.92$), suggesting that the potential of airborne PAH exposures at Pier 27 was similar among years and between these studies. There was no PAH measurement station in the Farr Island area to compare with PAH concentrations measured near Pier 27 (but see *Exposure differences at Hamilton Harbour sites*, Discussion).

In 2009, benzo[a]pyrene concentrations at Pier 25 exceeded the Ontario Ministry of Environment's 24-hour Ambient Air Quality Criteria (AAQC) guideline of 1.1 ng/m^3 for health effects (MOE 2008) during the potential exposure period 3/8 sampling periods, or 37.5% of the time (Figure 4). In 2010, benzo[a]pyrene concentrations exceeded the AAQC 2/8 (25%) during the potential exposure period. During both the 2009 and 2010 potential exposure periods, the two PAH with the highest mean concentrations of the 8 sampled PAHs were benzo[b]fluoranthene and chrysene (Figure 4). In 2010, the third most abundant PAH was benzo[k]fluoranthene, while in 2009 it was indeno[1,2,3-c,d]pyrene. In 2009, the highest PAH concentrations during the exposure period were on April 25, and during the 2010 exposure period, the highest PAH concentrations were recorded on March 21. Excluding the extreme outlier of March 21, 2010, when total PAH concentrations were approximately 7 times higher than the mean, hours of westerly wind (i.e. hours approximately downwind of the industrial core) was a moderately good predictor of total PAH concentrations for the 24 hour sampling period ($r^2 = 0.557$, $df = 1, 14$, $p = 0.0014$, $F = 16.41$). Even though it appears to cause Pier 27 to be directly downwind of the industrial core, southwesterly wind was not better correlated with PAH concentrations, so we did not examine it further.

PAH metabolites in cormorant tissues and samples

Benzo[a]pyrene-7,8-diol, a metabolite of the PAH benzo[a]pyrene, was detected in 4/19 bile samples, and 4/8 liver samples (Table 4; Figure 2). Quantification on the basis of wet weight was possible in all but one of these 8 samples (in this case the internal standard was not detected). Benzo[a]pyrene-7,8-diol was detected in samples from Farr Island and Pier 27, both in Hamilton Harbour, but not in the 2 samples from Mohawk Island, Lake Erie.

Differences in PAH metabolite concentrations among age/chick size would be difficult to evaluate with such a small sample size, but no patterns were obvious. We also detected a peak at the same mass-to-charge-ratio (m/z) as 2-hydroxynaphthalene, but a different retention time, which suggests it is not 2-hydroxynaphthalene. However, we cannot rule out the possibility it is the isomer 1-hydroxynaphthalene, or it could be a similar PAH metabolite. Alternatively, it could be an unknown contaminant inadvertently introduced from the general laboratory environment (e.g. Schlosser & Volkmer-Engert (2003)) or during sampling. These unknown peaks were detected in 10 samples (4 bile and 6 fecal) from all three sites (Appendix 1). We did not detect 1-hydroxypyrene, 2-hydroxy-phenanthrene, nor 1- or 3-hydroxybenzo[a]pyrene in plasma, fecal, bile, or liver samples from contaminated or reference site cormorants, although we could detect all except 1- or 3-hydroxybenzo[a]pyrene during spike and recovery (Figure 3). In bile samples, recovery of benzo[a]pyrene-7,8-diol was approximately 108%. Extraction blanks analyzed alongside samples were consistently below the detection limit for the compounds monitored.

Discussion

We found microsatellites mutations at the highest rate at Pier 27, 4.4 times higher than Farr Island and 7 times higher than Mohawk Island. We also found a metabolite of the PAH benzo[a]pyrene in some of the bile and liver samples taken from cormorant chicks in Hamilton Harbour, confirming that cormorants at Pier 27 and Farr Island are indeed exposed to and processing PAH contamination internally, and demonstrates their potential to have caused the observed microsatellite mutations.

PAH metabolites in cormorant tissues and samples

It is difficult to compare concentrations of benzo[a]pyrene-7,8-diol in samples from different sites, tissue types, sexes, or ages/weights of chicks given the low detection rate overall. However, while certainly not an extensive survey, we could not detect this metabolite in either of the Mohawk Island samples, which could suggest these cormorants are exposed to lower concentrations of PAHs. Samples in which we detected the metabolite did not appear to have higher masses, nor were they from larger chicks. Since fewer liver samples were analyzed but benzo[a]pyrene-7,8-diol was detected in four samples each of bile and liver, this could suggest that the liver would be a more promising tissue than bile for future work, which could be related to a lack of inhibitors, tissue structure, or the extraction method being more appropriate. The detection of benzo[a]pyrene-7,8-diol but not the other metabolites we screened for, such as 1-hydroxypyrene, 2-hydroxyphenanthrene, or 2-hydroxynaphthalene, was surprising given higher emissions of pyrene, phenanthrene, and naphthalene in Hamilton Harbour (Chapter One, Table 1). It is possible that we were examining a minor, rather than the major, metabolite of pyrene or phenanthrene in birds. The main determinant of body burdens may not be exposure but rather lipid solubility, tendency to bind to receptors or proteins, and ease of being metabolized, properties based on physical and chemical structure (Irigaray and Belpomme 2010). For example, of the PAH metabolites we screened for, benzo[a]pyrene-7,8-diol was the least polar, as evidenced by it having the longest column retention time (Figure 2). Perhaps this low polarity of benzo[a]pyrene is causing it to be retained in relatively higher concentrations in the cormorant bile and liver, as less polar compounds are more difficult to excrete and more likely to remain in the body (Amiard-Triquet & Rainbow 2011). Since

substituted and complexed PAHs are responsible for much biological action, perhaps a promising avenue would be to screen for their metabolites. Three and four ring alkyl PAHs such as C1-C4 phenanthrenes/anthracenes (with attached methyl groups) bioaccumulated in sea otters (*Enhydra lutris*) and their invertebrate prey (Harris et al. 2011), suggesting that targeting these or similar compounds in addition to hydroxy-PAHs could improve our low detection rates.

A wide variety of methods are used to detect PAH metabolites; our LC-MS/MS techniques may have been suboptimal and contributed to our lack of detection in many samples. For example, Troisi et al. (2006) found that a minimum of 5 grams of liver was required to detect any PAH metabolites in oiled guillemots; our liver samples used were much smaller due to different extraction methods. Freeze drying (lyophilization) of samples prior to extraction could be an improvement since it would allow for these larger samples (Troisi et al. 2006), but this substantially lengthens time required for analysis, as well as requiring specific equipment. We also observed suppression of the internal standard (bisphenol A) in several samples, suggesting that our guard column was not acting as a complete cleanup. A more sensitive instrumental setup could have helped to overcome our low detection rate; in the near future we aim to transfer our method to another instrument, along with investigating a wider variety of sample preparation optimization methods (e.g. Shahtaheri et al. (2006)).

Several studies have measured PAHs and/or their metabolites in various species of birds to date. Up to nine PAHs were quantified in muscle of Silver gulls (*Larus novaehollandiae*) and Australian pelicans (*Pelecanus conspicillatus*) from the contaminated Brisbane River estuary, Australia (Kayal & Connell 1995). Lesser scaup (*Aythya affinis*)

collected on the polluted Indiana Harbor Canal, US, showed higher total PAH concentrations than reference scaup from a game farm, and higher concentrations of phenanthrene and C1 naphthalenes (naphthalenes with one attached methyl group, Custer et al. 2000). Near a former refinery site in Wyoming, US, 12 PAHs were detectable in tree swallows (*Tachycineta bicolor*) and house wrens (*Troglodytes aedon*), while only one PAH could be detected in the same species at a cleaner reference site nearby (Custer et al. 2001). Double-crested Cormorants collected on the Houston Ship Channel (King et al. 1987) demonstrated temporal variability in their contaminant burdens, as eight PAHs were detected in birds collected during February while only two were found in those collected in November.

Very few papers have reported on metabolites of PAHs in birds, but an example concerns livers taken from wild guillemots (*Uria aalge*) previously oiled in spills (Troisi et al. 2006). Six PAH metabolites, including 1-hydroxychrysene, 2-hydroxyphenanthrene, 1-hydroxypyrene, and 1-hydroxybenzo[a]pyrene were reported. Concentrations (wet weight) ranged from 21 ng/g for 1-hydroxybenzo[a]pyrene to 391 ng/g for 1-hydroxychrysene. We attempted to detect several of those metabolites but they were all below detection limits. However, we did detect benzo[a]pyrene-7,8-diol in the same tissue type, liver, and the concentrations were within similar ranges (66-250 ng/g) as the oiled guillemots. In a dosing study with laying hens fed the PAHs phenanthrene, pyrene, and benzo[a]pyrene, Fournier et al. (2010) reported 2-hydroxyphenanthrene up to approximately 200 ng/g dry yolk weight, and 1-hydroxypyrene over 90 ng/g dry yolk. The concentrations of PAH metabolites in these two studies were similar to those we reported of benzo[a]pyrene-7,8-diol (wet weight) in livers and bile of cormorant chicks. A major difference is that these two previous PAH metabolite studies

used heavily contaminated birds (which had either died from oiling or were directly fed PAHs); environmental PAH concentrations in Hamilton Harbour may simply be lower than what would be required to reliably detect PAH metabolites in the majority of samples. Additionally, our study used very small chicks, while the previous studies used adults. It is possible that early in life, PAH metabolites are not reaching high concentrations as the hatched chick has been environmentally exposed to airborne particulate for only a few days. Despite our low rate of detection, this is the first known report of PAH metabolites in wild birds not directly oiled but rather exposed to ambient chemical contamination.

Chick-parent mismatches

Cormorants may lay eggs in nests of other cormorants (Hatch & Weseloh 1999) or nests of species such as Herring Gulls (Somers et al. 2011). Rates of extrapair paternity have not previously been studied, but in the shag *P. aristotelis* 18% of 28 chicks showed extrapair paternity (Graves et al. 1992) while in the shag *P. atriceps*, Calderón et al. (2011) found no cases of extrapair paternity nor intraspecific brood parasitism/adoption in 37 families. In studies such as this where various combinations of extrapair paternity/maternity or displacement are possible, differentiating between mutations and these events is critical. We identified five chicks whose genotypes were consistent with intraspecific brood parasitism, in that one chick within a nest of multiple matching chicks did not match *either* parent. Alternatively, although we attempted to sample nests with non-mobile chicks, some chicks may have been large enough to make it to a nearby nest and these could therefore represent cases of chick displacement or adoption. Furthermore, in two families, just one chick within a family of multiple chicks mismatched the putative father, but matched the mother, suggestive

of extrapair paternity. This would result in rates of approximately 2% of all chicks genotyped in this study being products of brood parasitism or displacement, and 0.8% of all chicks resulting from an extrapair paternity. These would represent conservative estimates since in some cases where it was not possible to distinguish between incorrect trapping and an extrapair event, we assumed a trapping issue. Despite a lack of previous data, (Hatch & Weseloh 1999) suspected that Double-crested Cormorants would have low extrapair paternity; our data provide the first evidence for extrapair paternity and intraspecific brood parasitism or adoption in this species. While this result was interesting, it also points to possible problems when using a wild, free-ranging species in a mutation study. Beck et al. (2003) discuss the benefits of a microsatellite mutation study in a population with almost all individuals banded and tracked throughout multiple generations, which allows for verification of mutated alleles based both on previous parents and also further offspring. However, a technique such as this would only be usable in rare circumstances where a long-term study already exists, and is unfortunately not reasonable for waterbirds in colonies containing thousands of adults.

An additional problem revealed after genotyping putative cormorant families was the incorrect trapping of one or both adults. The potential for this error was demonstrated in the one instance when we accidentally re-set the trap on a nest where two adults had already been sampled, but then shortly after caught a third adult. This probably results from adult cormorants walking and hopping quickly through other nests in the colony while returning to their own nests, and accidentally triggering the trap. Since there is no known way to reduce this type of trapping error, researchers should be careful to aim for a greater sample size than what they estimate they would need to demonstrate mutation induction, since a relatively high

proportion of families may have to be excluded after genotyping (14% in this study). Additionally, these extra families increased the resources needed (i.e. time and money) without increasing the power of the study since they could not be used for statistical analysis of mutations. One potentially useful approach to minimize these errors would be to regularly reset a trap on the same nest again after both putative parents have been caught. If a third adult bird is caught, that third adult can also be genotyped and assessed for the possibility that it is a correct parent. However, while reducing the proportion of excluded families, this could also increase time and money required for genotyping if up to 50% more parents will be genotyped.

Mutation rates and models

Our data indicate that microsatellite mutations in cormorants were differentially induced among three colonies with varying degrees of PAH contamination. Several lines of evidence indicate our mutation results and conclusions are robust. We used an extremely conservative method for removing families based on shared alleles, and even when families were removed due to a higher proportion of allele mismatches, the proportion of removed families was much higher at the Hamilton Harbour sites. When assessing the mutations statistically, we considered cases where the same mutation was found in two chicks from the same family as a single mutation, since they may be correlated and result from the same gametogenic event. Since these two 'double' or non-unique mutations were both at Pier 27, if we considered each mutation to be independent, as in Anmarkrud et al. (2011), the number of mutations at the most contaminated site would have been even higher. We considered cases where there are two mutations in one family (but in different loci) to be two separate mutations, since these

two mutations result from entirely different gametogenic events. In one case this even appears to result from two different parents as one mutation is from the paternal side and the other from the maternal line; in another case, this is also possible as the second mutation could have come from either parent (Table 2). However, it is true that if we had considered the family as simply one unit for mutation, this would have reduced the significance of our result. The GEE approach discussed previously would have been one way to address this issue, by counting two chicks in one family with different mutations as a sample size between 1 and 2; in the future, we may consider different ways to analyze this mutation data, since such a wide variety of techniques are available and new statistical approaches for this type of data are continually being refined.

Mutation rates vary enormously across locus type (e.g. tandem repeat DNA versus coding DNA), sex, species, and tissue type, and age (e.g. Dubrova et al. (2006); May et al. (2000), Hardwick et al. (2009)). Environmental stressors such as radiation and certain chemicals have repeatedly been shown to increase tandem repeat DNA mutation rates (e.g. microsatellites in radiation-exposed medaka (*Oryzias latipes*): 1.65×10^{-2} per locus per offspring (Tsyusko et al. 2007); ESTRs in mice exposed to alkylating agents and topoisomerase inhibitors: 0.05 to 0.183 in the paternal germline, Vilariño-Güell et al. (2003)). Background microsatellite mutation rates are generally in the range of 10^{-7} to 10^{-3} per locus per generation (Buschiazzo & Gemmell 2006). In birds, background microsatellite mutation rates ranged from 2.96×10^{-3} (lesser kestrels, *Falco naumanni*; Ortego et al. (2008)) up to 1.4% of all meioses (superb fairy-wren, *Malurus cyaneus*; Beck et al. (2002) and 10.8% of all meioses (sand martins *Riparia riparia*; Anmarkrud et al. (2011)). Per-individual microsatellite

mutation rates in this study ranged from 0.004 to 0.012. The five loci mutated at different rates, with the highest rates in COR 03 and COR 06, which also had the largest numbers of alleles (17 and 15, respectively Table 1). This appeared to partially support the idea that mutation rate can be positively correlated with the number of repeat units, at least in perfect microsatellite loci (Primmer & Ellegren 1998). However, the perfect locus CORM 4A had the highest number of repeats (18) and a lower number of alleles (12) and did not show as many mutations as COR 03 and COR 06. Interruptions in the repeat sequence probably reduce or eliminate mutation (Primmer & Ellegren 1998), and we found some support for this idea in that the imperfect loci COR 01 and CORM 5A2 had low numbers of mutations identified (1 and 2 respectively).

Not all of the mutations we identified were consistent with the stepwise mutation model in that 6/11 unique mutations showed a change of 1 repeat unit (evenly split between gain and loss). The remaining five mutations demonstrated larger multi-repeat-unit changes that were inconsistent with a stepwise mutation model (e.g. Nest 53, Table 2), including a loss of 2 repeat units (2), gain of 3, gain of 4, and loss of 4. However, the problem remains that to even designate this change in repeat units, we had to rely on the stepwise model to identify the putative contributing allele and parent. Given the other available models, such as the infinite alleles model, two-phase stepwise model, K-allele model, and the newer life cycle model (Buschiazzo & Gemmell 2006), the stepwise model may not best explain microsatellite evolution and mutation. In the life cycle model, expansion (i.e. gain of repeat unit(s)) is more common in the beginning of the growing microsatellite's existence, while contraction (i.e. loss of repeat unit(s)) predominates in the later phases, towards the 'death' or disappearance of

the microsatellite region as it accumulates point mutations, substitutions, and insertions that render it less and less mutable (Buschiazzo & Gemmell 2006). Therefore, what might be more important in determining gains versus losses is not necessarily length or repeat type, but age of the microsatellite, which in our cases is unknown. Keirle et al. (2011) demonstrated non-stepwise microsatellite mutations in fungus, while Macdonald et al. (2011) showed that male wallabies produce sperm with large multi-unit repeat changes in microsatellites, providing strong evidence against the stepwise mutation model. Complex microsatellite mutations were demonstrated in three *Drosophila* fruit fly species (Colson & Goldstein 1999), where perfect and imperfect microsatellites demonstrated different mutational patterns. In various species of birds, Primmer & Ellegren (1998) suggested that imperfect and perfect microsatellites mutated quite differently, and since our study included both types we may be in fact looking at different models of microsatellite mutation.

Buschiazzo & Gemmell (2006) highlighted additional studies where multi-step mutations seemed to predominate, in species as diverse as lizards, snails, and humans, suggesting that some loci and/or alleles may be more likely than others to produce this type of mutation. However, our data were not suggestive of this trend as they demonstrated a mix of multi-repeat-unit mutations and single step mutations within the same loci (e.g. COR 06). Our data support the accumulating evidence that the dynamics governing mutations are complex and should probably not be modelled with overly simplistic yet appealing models (Keirle et al. 2011).

Sex biased mutations

It was not always possible to ascribe a parent to the mutation. In the remaining cases, we could identify one allele as belonging to one parent such that the remaining mutant allele must have come from the other parent. Of the cases where we identified the mutating parent, only 33% of mutations appeared to arise from the male germline. Many studies have reported a higher proportion of germline mutations derived from the paternal line than from the maternal lineage (e.g. (Somers et al. 2002; 2004) and the theory of male-biased mutations remained dominant for many years, stemming from the ideas that males have increased DNA replication as an adult and smaller gametic investment. However, on the contrary, a bias towards maternally derived microsatellite mutations have recently been reported in a variety of species and loci, such as in olive ridley sea turtles (Hoekert et al. 2002), superb fairy-wrens (*Malurus cyaneus*, Mcy μ 8 locus; Beck (2003), lesser kestrels (*Falco naumanni*; Ortego et al. (2008), and five species of *Hirundinidae* swallows (Anmarkrud et al. 2011). Additionally, some have showed no significant bias such as the Mcy μ 4 locus in superb fairy-wrens (Beck 2003). This variety of results demonstrates that biases are not consistent across taxa or even loci within a species and the finding of high proportion of female derived mutations in cormorants is not unprecedented.

Sex-biased mutations are thought to be linked to sperm competition, such that males in species with mating systems that include polyandry will produce higher numbers of sperm, thus increasing the probability of mutations (Anmarkrud et al. 2011; Ellegren 2007). If this is true, mice could indeed display male-biased mutations while socially monogamous cormorants, with relatively low levels of sperm competition, may not follow this pattern at all. Interestingly, there are also fewer spermatogonial cell divisions in birds than in mammals

(Pudney 1995) so this probably provides relatively fewer opportunities for the male germ line to mutate.

Exposure differences at the three colonies

This study evaluates the effects of a contaminated environment (including both air and water/diet), where cormorants could have been exposed via these two main routes. To assess the possibility that differing diets at the two Hamilton Harbour sites could contribute to differential contamination uptake via the food web, we conducted a concurrent study using both traditional diet analysis and fatty acid analysis (Chapter Three). Cormorant diets at the two sites in Hamilton Harbour were similar as estimated with regurgitate, consisting almost entirely of Alewife (*Alosa pseudoharengus*, 51-56% by frequency) and Round Goby (*Neogobius melanostomus*, 25-42% by frequency). Fatty acid profiles at these two colonies were also extremely similar (Chapter Three). Cormorants make use of wide foraging ranges, typically several kilometres from their home colony (Anderson et al. 2004; Hatch & Weseloh 1999). This suggests that adult cormorants from the Pier 27 and Farr Island colonies, which are approximately three kilometres from each other, would draw on the same food resources in the general Hamilton Harbour and Lake Ontario area. Additionally, since cormorants are largely piscivorous and eat only natural food sources, cormorants from the two Hamilton Harbour colonies do not ingest contaminated human food. These multiple lines of evidence suggest that contamination via diet at these Hamilton Harbour sites would be similar, and that therefore the major differences between the two Hamilton Harbour sites probably lies in their airborne exposure.

Farr Island was subject to fewer hours downwind of industry than was Pier 27 during study years. The proportion of time that Farr Island was downwind may be a less accurate measure since it is farther from the industrial area, and therefore more subject to variation in exposure since there is more time and area for pollutants to diffuse out into before reaching the opposite site of the Harbour and Farr Island. Despite the lack of a monitoring station year-round at Farr Island, Wallace et al. (2009) used mobile monitoring vans, covering the areas near Farr Island and Pier 27, and demonstrated differences between their exposure to both traffic and industrial emissions. A detailed examination of their results can help to distinguish between emission types affecting these two sites (Wallace et al. (2009)). NO_x (nitrogen oxides) are an indicator of traffic pollution, and the areas around both Pier 27 and Farr Island receive similar concentrations under prevailing southwest wind conditions (air closest to both sites approximately 51-100 ppb NO_x, Fig. 5, Wallace et al. (2009)). When wind blows from the northeast, Farr Island is subject to similar if not *more* traffic-based contamination (air closest to Pier 27 with 0-50 ppb NO_x, air closest to Farr Island with 201-400 ppb NO_x, Fig. 6, Wallace et al. (2009)). However, SO₂ concentrations, which track industrial emissions, show a different pattern. Under both southwesterly and northeasterly winds, air around Pier 27 has much higher concentrations of SO₂ than air around Farr Island (Figs. 3 and 4, Wallace et al. (2009)). With southwesterly winds, air around Farr Island generally received 20-40 ppb SO₂, while Pier 27 was near air with 41-80 ppb SO₂ (Fig. 3, Wallace et al. (2009)). With northeasterly winds (Fig. 4, Wallace et al. (2009)), air around Farr Island contained 11-20 ppb SO₂, but at Pier 27 SO₂ was very high, at 81-249 ppb (Wallace et al. 2009). Therefore, Pier 27 is subject to much higher industrial emissions than is Farr Island. Since the majority of mutations were found at Pier 27 and the mutation rate was 4.4 times higher than that at Farr Island, this suggests that

airborne industrial and not traffic-related contaminants were more likely to have induced these mutations.

PAH exposure and mutation induction

Our results support the conclusions of previous mutation studies in Hamilton Harbour (Yauk & Quinn 1996; Yauk et al. 2000; Somers et al. 2002, 2004; Yauk et al. 2008) that have suggested with increasing certainty that airborne pollution in Hamilton Harbour is associated with increased mutation in tandem repeat DNA loci, and that industrial contaminants including PAHs are likely responsible.

PAH concentrations in Hamilton Harbour near the Pier 27 site were high and regularly exceeded the provincial health effect guidelines. The 8 PAHs measured in Hamilton Harbour are all associated with the particulate (rather than the vapour) phase (Sanderson et al. 2004), demonstrated to contain the mutation-inducing fraction for caged mice in Somers et al. (2004). Total PAH concentrations during the years of this study were similar to Yauk et al. (2008) and Somers et al. (2004), suggesting that the potential of airborne exposures at Pier 27 was similar among years and studies. However, 2009 and 2010 showed higher variability, with total PAH concentrations up to 58 ng/m³ on one day in March 2009; PAH concentrations were therefore higher at this one point than in any other day recorded during these previous studies. Wind patterns and therefore PAH concentrations and exposure in Hamilton Harbour can be complicated, with frequent pollution-trapping temperature inversions (Wallace et al. 2010a,b) and lake effect winds coming from the northeast (Wallace et al. 2009); the very high PAH concentration during one day of the cormorant breeding season may have contributed to mutation induction.

PAH mitigation efforts for the submerged coal tar Randle Reef in Hamilton Harbour are scheduled to begin soon. Declining airborne emissions from steel production in Hamilton Harbour are also changing airborne PAH emissions over the long term. This could provide a variety of interesting and relevant opportunities to temporally study mutation induction in both terrestrial and aquatic species. Drawing on the expertise gained in caged fish and mice studies in Hamilton Harbour, the use of caged experimental birds such as zebra finches (*Taeniopygia guttata*) could represent a useful bird model for Hamilton Harbour without the previously discussed drawbacks we experienced that are possible when using free-ranging wildlife.

Exposure relative to timing of gamete development and embryogenesis is important. When mice are exposed to radiation before sperm meiosis, the resulting minisatellite mutations are thought to be true germline mutations resulting from mutations induced during spermatogenesis (Yauk & Polyzos 2005). However, when mice are exposed after sperm meiosis, the resulting minisatellite mutations would have had to be induced in early embryogenesis since spermatozoa “lack any activity of DNA metabolism” and therefore cannot mutate (Niwa 2003). If this is also true for our study, the induced mutations we observed could be a mix of both types of mutations, since adult cormorants were exposed continuously. However, a mutation in early embryogenesis results in a visible mosaicism, with 3 alleles at one locus (Bouffler et al. 2009; Burr et al. 2006). We observed only two alleles at each of the mutated loci, which is a good indication that these are true germline (rather than somatic) mutations. Therefore, mutations were likely induced in gametes, such that sperm and egg (rather than embryo) exposure would be the most relevant to mutation. Sperm

development in birds is much faster than in mammals, operating on a 12-13 day cycle, with spermiogenesis (the process that produces spermatozoa from spermatids) at the last few days of this process (Aire 2007). Chicks with mutations were sampled from May 17th to July 7th, such that those eggs were laid around the beginning of April to the beginning of June, since chicks hatch approximately 30 days after eggs (Hatch & Weseloh 1999). This means that spermatids undergoing meiosis would have been exposed to chemical contamination in Hamilton Harbour during the months of March, April, and May. However, since we did not find a male-biased mutation rate (assuming a stepwise model), the development of eggs in female cormorants could be more relevant to this study. Eggs could have developed mutations during the beginning of the female cormorant's lifetime, as the process of oogenesis is complete at the latest four days after hatching (Hughes 1963; Brambell 1926). Since females show some fidelity to location year to year, even under disturbance (Scherr et al. 2010) and female Great Cormorants (*P. carbo sinensis*) showed strong philopatry towards their natal sites (Schjørring 2001), it is possible that females have returned to the same colony every year and their exposure at Hamilton Harbour sites would have remained roughly consistent since their birth. Mutations could also have been induced in the final meiotic divisions of the oocyte in the beginning of April to the beginning of June. Alternatively if these mutations were induced in early embryogenesis, these mutations would have been induced shortly after eggs were laid, from the beginning of April to the beginning of June.

In understanding mutation mechanisms, it is also important to consider the effects of single versus chronic exposure and their effects on mutation mechanisms (Yauk 2004). The lifetime exposure of these wild cormorants is quite different from the 10 weeks used for mice

in Hamilton Harbour by Somers et al. (2004). While we only examined PAH contamination in two years, the cormorants sampled in this study have probably been exposed to similar or higher concentrations of PAHs several months per year for their entire lives, as well as other mutagens. With short or single exposures, the body's mutation repair mechanisms may be able to overcome this toxic insult, but regular exposure over many years may contribute differently in ways we do not fully understand. The mean adult life expectancy of cormorant is 6 years but they can live up to 17, with breeding beginning around the third year (Hatch & Weseloh 1999), so multiple years in Hamilton Harbour may have been continually and progressively altering the DNA of these cormorants.

Environmental microsatellite mutations

Previous research demonstrated significantly increased microsatellite mutation rates in barn swallows breeding near Chernobyl (Ellegren et al. 1997; Møller et al. 2007) although Smith (2008) discussed alternate explanations for these findings, including swallow population reductions in abandoned radioactive areas. Ben-Shlomo & Shanas (2011) reported extra peaks in microsatellite loci of wild mice breeding in an asbestos-contaminated area of Israel, which they suggested could have arisen through somatic mutation. However, Chapter 2 of this thesis is the first evidence for germline microsatellite mutations induced through exposure to non-radioactive chemical environmental contamination. Microsatellites could therefore represent a useful tool in future environmental mutation studies, especially since they are available for many species and relatively easy to amplify.

These results also contribute to the ever-increasing body of studies which have shown effects from environmental pollution that contains PAHs on wildlife (e.g. Miles et al. 2007; Fair

et al. 2010; Custer et al. 2000, 2001; Kayal & Connell 1995; Harris et al. 2011) and humans (Pavanello et al. 2010; Okona-Mensah et al. 2005; Valavanidis et al. 2008; Jacob & Seidel 2002; Laumbach & Kipen 2010; Besaratinia & Pfeifer 2008; Perera et al. 2005; Perera et al. 2009). Practically all species and environments worldwide are exposed to air pollution, and with its high mutagenic and carcinogenic potential, the need for future studies to detail its varied effects on DNA is great.

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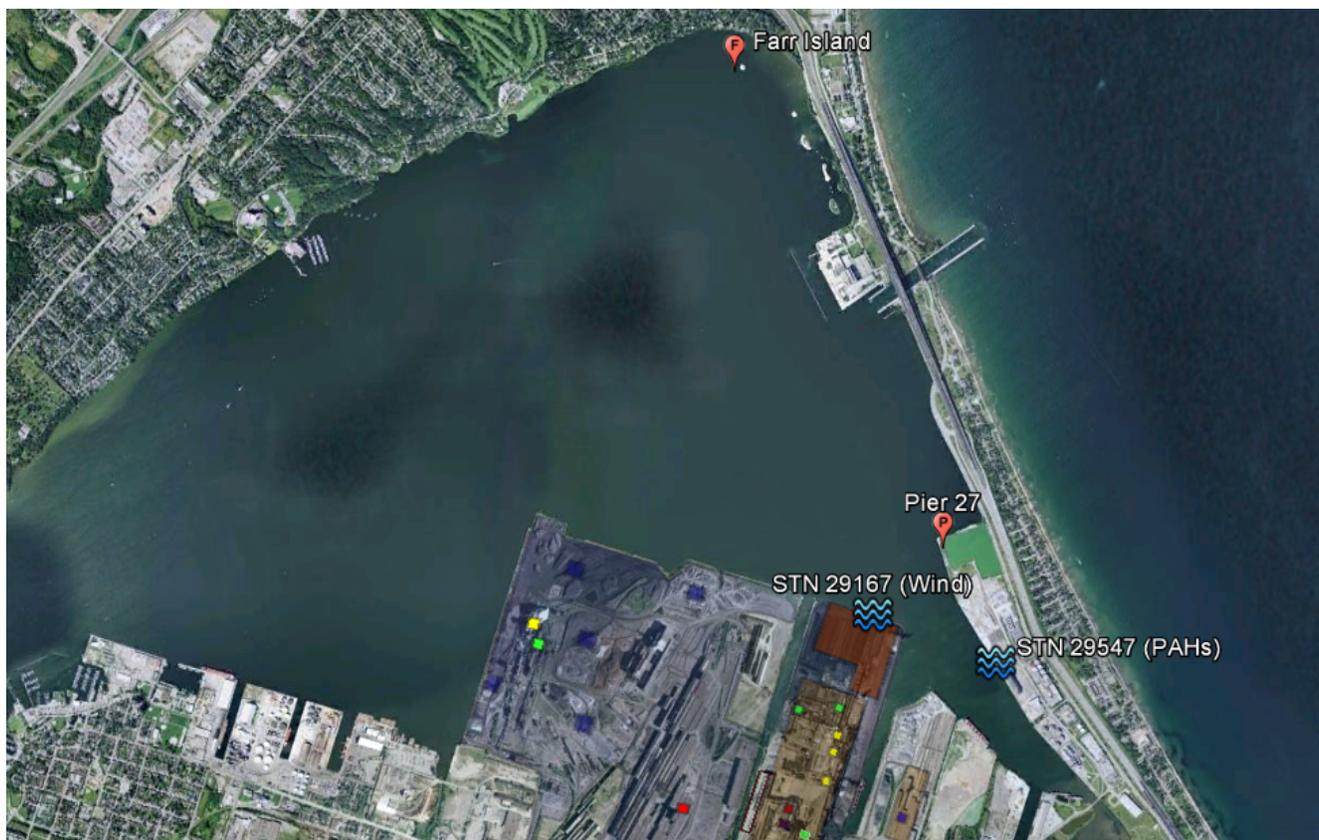


Figure 1. Hamilton Harbour, western Lake Ontario, Canada. Cormorant colony study sites indicated with red placemarks, and monitoring stations indicated with blue wind icons. Both stations belong to the Hamilton Air Monitoring Network (hamnair.ca). Colours in the Hamilton industrial area represent various industrial steel production processes; areas producing chemicals most relevant to mutation induction are coloured green for cokemaking (4 areas) and yellow for ironmaking (4 areas, included with permission from Environment Hamilton's Stackwatch Interactive Map, <http://www.goodneighbourcampaign.ca/stackwatch>). Base images DigitalGlobe, TerraMetrics 2012, Google Earth.

Table 1. Details of microsatellite primers used in this study. Fluorescent tags included are HEX (green), 6-FAM (blue), and NED (yellow). k, number of alleles, H_o, Observed Heterozygosity; H_e, Expected Heterozygosity.

Locus	Repeat Unit	Repeat Type	Product Length	k	H _e	H _o	Primer sequences (5' → 3') and fluorescent tag locations
COR 01	(GATA) _{4,7}	Imperfect: interrupted	155 - 203 bp	12	0.763	0.766	F: 6-FAM/TCATGAACCAGCACCACATAG and NED R: TTGATAGGCAGGTGACATGG
COR 03	(TATC) ₁₇	Perfect	180 - 246 bp	17	0.882	0.891	F: 6-FAM/TGCAGTTGCATTCCCTTTC R: CTTCTAATGTGTTACTACTGTCTGG
COR 06	(TATC) ₁₅	Perfect	124 – 181 bp	15	0.875	0.922	F: GCCCACCATTCTTATTGCAC R: 6-FAM/AGAAAGAATTGAGCCTAAGAAGTGA
CORM 4A	(TGAG) ₁₈	Perfect	170 - 214 bp	12	0.888	0.922	F: HEX/TTGTGGTCCAGTTTCAGGTG R: TGCTCAGTTGCTTTTCTCACA
CORM 5A2	(AGAC) ₈ (AGAT) _{6,8}	Imperfect: compound, interrupted	228 – 276 bp	13	0.832	0.862	F: GTGTTGTGTCAGGTGGCTG R: HEX/TGGTGCCCTGCTTTTGTG

Table 2. Details of microsatellite mutations observed in cormorant families. Mutations were observed at all of the three sites and at 5 of 5 microsatellite loci used. Mutated alleles in chicks and putative parental progenitor allele(s), assuming closest allele contributed (stepwise mutation model), shown in bold.

Site	Family ID	Chick ID	Locus	Paternal Alleles (bp)	Maternal Alleles (bp)	Chick Alleles (bp)	Unit Change	Parental Origin
Pier 27	Nest 27	PHA27	03	184 /226	184 /226	184/ 189	+1	Either
		PHA28				189 /226		
Pier 27	Nest 36	PHA296	06	153/165	137/ 165	153/ 161	-1	Maternal
Pier 27	Nest 53	PHA565	06	149/169	145 /169	128 /169	-4	Maternal
		PHA566				128 /169		
Pier 27	Nest 54	PHA569	03	188 /204	218/226	180 /218	-2	Paternal
			06	128/157	128/ 161	128/ 173	+3	Maternal
Pier 27	Nest 55	PHA572	5A2	244/248	232/ 240	244 /248	+1	Maternal
Pier 27	Nest 68	PHA511	4A	174/194	174/ 178	169 /174	-2	Maternal
		PHA513	5A2	232/228	232/ 256	232/ 252	-1	Maternal
Farr I.	Nest 153	PHA226	4A	174/ 182	202/206	198 /202	+4	Paternal
			01	179/ 183	179/ 183	179/ 187	+1	Either
Mohawk I.	Nest 212	PHA595	03	205 /226	222/230	200 /230	-1	Paternal

Table 3. Locus-specific, site-specific, and total mutation rates and numbers of mutated alleles in cormorant families. Each microsatellite locus was amplified for adults and chicks of 97 families (248 chicks total): 39 from Pier 27 (83 chicks), 30 from Farr Island (92 chicks), and 28 from Mohawk Island (73 chicks). “Mutation rate (indiv.)” is the per-individual mutation rate and “Mutation rate (allele)” is the per-allele mutation rate (mutation rate per meiosis).

Site	Pier 27 Hamilton Harbour	Farr Island Hamilton Harbour	Mohawk Island (reference)	Total (all three sites)
COR 01 (imperfect)				
# of mutated alleles	0	1	0	1
Mutation rate (indiv.)	-	1/92 = 0.0108	-	1/248 = 0.0040
Mutation rate (allele)	-	1/184 = 0.0054	-	1/496 = 0.0020
COR 03 (perfect)				
# of mutated alleles	3 (2 unique)	0	1	4 (3 unique)
Mutation rate (indiv.)	2/83 = 0.0240	-	1/73 = 0.0136	3/248 = 0.0120
Mutation rate (allele)	2/166 = 0.0120	-	1/146 = 0.0068	3/496 = 0.0060
COR 06 (perfect)				
# of mutated alleles	4 (3 unique)	0	0	4 (3 unique)
Mutation rate (indiv.)	3/83 = 0.0361	-	-	3/248 = 0.0120
Mutation rate (allele)	3/166 = 0.0180	-	-	3/496 = 0.0060
CORM 4A (perfect)				
# of mutated alleles	1	1	0	2
Mutation rate (indiv.)	1/83 = 0.0120	1/92 = 0.0108	-	2/248 = 0.0080
Mutation rate (allele)	1/166 = 0.0060	1/184 = 0.0054	-	2/496 = 0.0040
CORM 5A2 (imperfect)				
# of mutated alleles	2	0	0	2
Mutation rate (indiv.)	2/83 = 0.0240	-	-	2/248 = 0.0080
Mutation rate (allele)	2/166 = 0.0120	-	-	2/496 = 0.0040
Total (all five microsatellite loci)				
# of mutated alleles	10 (8 unique)	2	1	13 (11 unique)
Mutation rate (indiv.)	8/83 = 0.0963	2/92 = 0.0217	1/73 = 0.0136	11/248 = 0.044
Mutation rate (allele)	8/830 = 0.0096	2/920 = 0.0021	1/730 = 0.0013	11/2480 = 0.0044

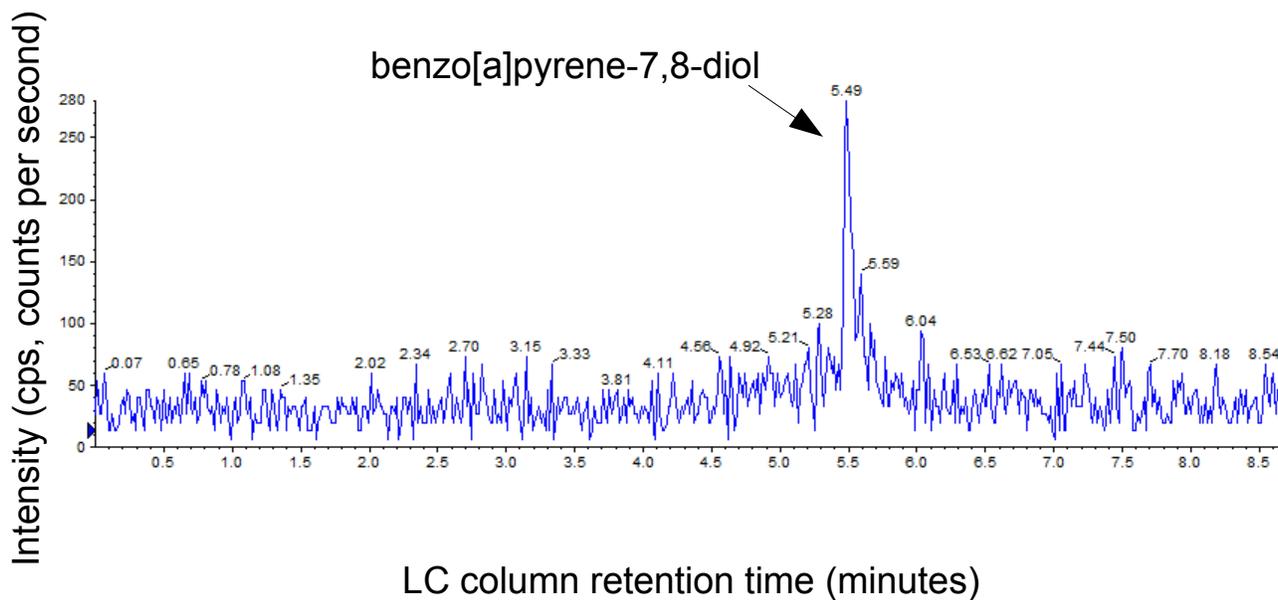


Figure 2. LC-MS/MS chromatogram for benzo[a]pyrene-7,8-diol, a Phase I metabolite of the PAH benzo[a]pyrene, extracted from Double-crested Cormorant bile (Chick 24, 66.84 ng/g wet weight – see also Table 4).

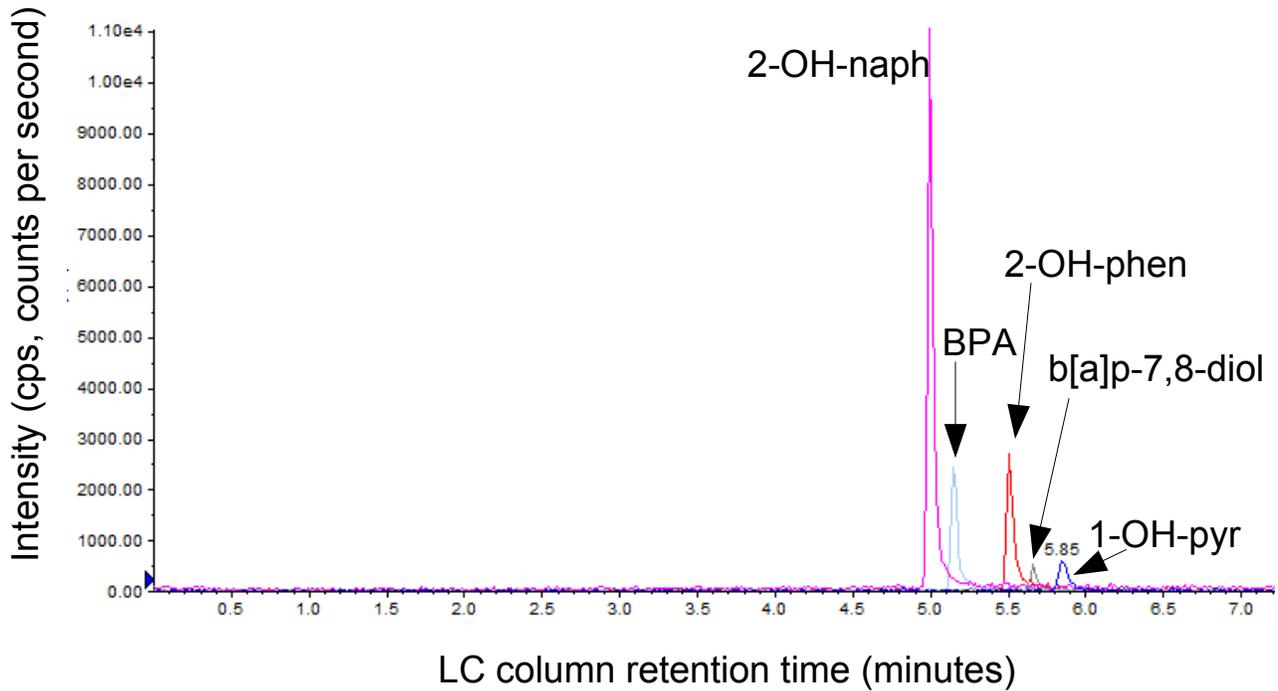


Figure 3. LC-MS/MS chromatogram for PAH metabolite spike and recovery of a cormorant chick bile sample. The x axis represents the liquid chromatography (LC) column retention time in minutes, i.e. the time the analyte took to flow through the chromatography column, which is relative to the polarity of the analyte. The y axis represents the intensity of the response from the detector in counts per second (cps). Neither 1- nor 3-hydroxybenzo[a]pyrene could be detected during spike and recovery. Abbreviations: 2-OH-naph: 2-hydroxynaphthalene, BPA: bisphenol A (internal standard), 2-OH-phen: 2-hydroxyphenanthrene, 1-OH-pyr: 1-hydroxypyrene, b[a]p-7,8-diol: benzo[a]pyrene-7,8-diol.

Table 4. Benzo[a]pyrene-7,8-diol PAH metabolites detected in cormorant chick liver and bile tissues by LC-MS/MS. 18 bile samples were screened – 9 from Pier 27, 7 from Farr Island, and 2 from Mohawk Island. 8 liver samples were screened – 4 from Pier 27, 3 from Farr Island, and 1 from Mohawk Island.

Chick ID and weight	Site	Sample type used	Wet weight of liver or bile used (g)	Concentration [ng/mL] in sample vial	Concentration [ng/g] in wet weight of sample
21 (42 g)	Farr Island	bile	0.039	4.52	115.89
		liver	0.094	23.5	250.00
24 (56 g)	Farr Island	bile	0.187	12.5	66.84
		liver	0.378	29.7	78.57
30 (40 g)	Pier 27	liver	0.311	50.6	162.70
31 (41 g)	Pier 27	liver	0.229	34.7	151.53
32 (54 g)	Pier 27	bile	0.059	unable to quantify	
35 (61 g)	Pier 27	bile	0.058	3.82	65.86

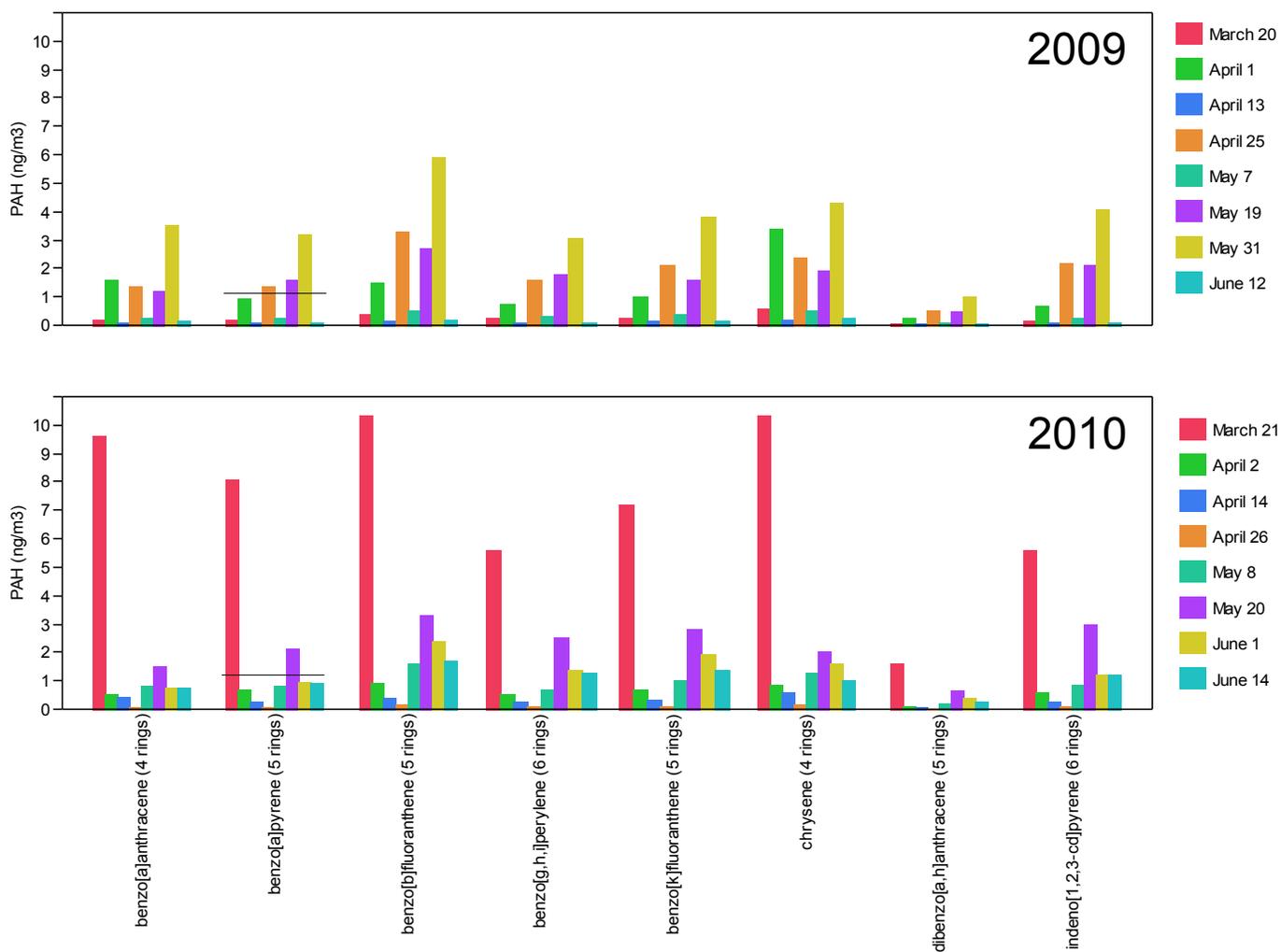


Figure 4. 8 airborne PAHs measured by GC/MS at the Pier 25 HAMN monitoring site (Station 29547, 1.2 km southeast of Pier 27, Hamilton Harbour), during the potential exposure period for breeding cormorants, March 20 – June 14 in both 2009 and 2010. Y axes are both scaled to 11 ng/m³ PAH to allow for easier visual comparison between graphs. The horizontal black line over the benzo[a]pyrene bars represents the Ontario Ministry of Environment Ambient Air Quality Criteria for benzo[a]pyrene in a 24 hour period, at 1.1 ng/m³ (the other 7 PAHs presented have no set criteria).

Table 5. Hours downwind in three Hamilton Harbour mutation studies, grouped into the 'Hours downwind during 24 hour interval' categories used by Somers et al. (2004) and subsequently Anmarkrud et al. (2011). Wind data for cormorant research in 2009 and 2010 is from Station 29167 of the Hamilton Air Monitoring Network (HAMN, hamnair.ca). 'Wind direction' refers to the location that wind originates from, such that it causes the field site to be downwind of the industrial area. W refers to a westerly wind (180° to 360°, which causes Pier 27 to be downwind of the industrial area). S refers to a southerly wind (90° to 270°) causing Farr Island to be downwind of the industrial area, WNW refers to the 270° to 315° wind span used by Anmarkrud et al. 2011 which caused their site to be downwind of the industrial area.

Study	Somers et al. 2004	Anmarkrud et al. 2011	This thesis (King 2012)			
Species	mice	tree swallows	cormorants			
Exposure year	2000	2008	2009	2010	2009	2010
Exposure days	70	30	87	87	87	87
Site	Pier 25	Windermere Basin	Pier 27		Farr Island	
Wind direction	W	WNW	W	W	S	S
Hours downwind	% exposure days in each category					
0	13	40	14	14	16	16
1 to 3	16	20	16	14	15	20
4 to 9	14	33	17	19	37	27
10 to 18	20	7	27	23	24	33
19 to 23	13	0	2	15	3	3
24	24	0	24	15	5	1

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Chapter Three: Fatty acid profiles and regurgitated food samples reveal diet differences between Lake Ontario and Lake Erie Double-crested Cormorants (*Phalacrocorax auritus*)

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Abstract

Double-crested Cormorants (*Phalacrocorax auritus*) living in highly polluted Hamilton Harbour, Ontario, Canada may be exposed to environmental contaminants through their piscivorous diet. We compared cormorant diets at two sites in Hamilton Harbour, western Lake Ontario, and one site in northeastern Lake Erie using both regurgitated samples and fatty acid analysis to assess whether diets varied in ways that could promote differences in contaminant uptake among these three colonies. Cormorant diets at the two sites in Hamilton Harbour were similar as estimated with regurgitate, consisting almost entirely of Alewife (*Alosa pseudoharengus*, 51-56% by frequency) and Round Goby (*Neogobius melanostomus*, 25-42% by frequency). Fatty acid profiles at these two colonies were also extremely similar,

suggesting that contamination via diet at these Hamilton Harbour sites is likely similar. A higher number of species of fish were identified in regurgitate from cormorants at Mohawk Island, Lake Erie, but overall diets were dominated by Round Goby (70% by frequency). Fatty acid profiles indicated large differences between Hamilton Harbour and Mohawk Island diets, but only very small differences between the two Hamilton Harbour sites. PMI (polymethylene-interrupted) fatty acid concentrations and percentages were much higher in Lake Erie cormorant samples, indicating those cormorants consumed higher amounts of prey that feed on invasive zebra and quagga mussels (*Dreissena* spp.). Dreissenid mussels bioaccumulate various pollutants, and can transfer them to fish and upwards through food webs. However, since contaminant concentrations are much lower in northeastern Lake Erie than Hamilton Harbour, cormorants at Mohawk Island are likely taking up lower levels of contaminants through diet compared to those from the two Hamilton Harbour sites.

Introduction

Hamilton Harbour, a 2150 hectare embayment at the west end of Lake Ontario, is one of the most polluted areas on the Great Lakes (Hall et al. 2006; Zeman & Patterson 2003; Marvin et al. 2000, 2002; Sofowote et al. 2008, 2010a, 2010b). Emissions from industry, including Canada's two largest integrated steel mills, and diesel and gasoline emissions from nearby highways are major contributors to air and sedimentary contamination in the Harbour (Sofowote et al. 2008). The Harbour supports a large breeding population of Double-crested Cormorants (*Phalacrocorax auritus*) that may be affected by both airborne and dietary contamination. Contaminants transferred through diet have been reported for this species

(Somers et al. 1993), and other piscivorous Great Lakes waterbirds such as common terns (*Sterna hirundo*) (Ward et al. 2010), and cormorants are susceptible to environmental pollution (Ludwig et al. 1996; Fox 2001). Cormorants feed on fish species that eat invasive dreissenid mussels (Marentette et al. 2010; Somers et al. 2003) that can bioaccumulate and subsequently transfer contaminants (and their harmful metabolites) to fish (Custer & Custer 2000). Therefore, cormorants that 'secondarily' consume higher proportions of mussels might be exposed to more contamination through their diet. An exploration of how variation in cormorant diet could promote differences in contaminant uptake was necessary, especially in contaminated Hamilton Harbour.

Years of environmental and ecological damage (reviewed in Dermott et al. (2007)) have resulted in a highly degraded fish community in Hamilton Harbour, with a much lower percentage of native specialist fish than a healthy ecosystem would support (Brousseau & Randall 2008). Numerous introduced species have continued to change the fish communities. This may influence cormorant diets in western Lake Ontario, partially because the total number of species available in the area is relatively low, and most native fish species' populations are too low to make up a large portion of the diet. Reflecting this low diversity and dominance of introduced species, the diet of cormorant chicks in Hamilton Harbour was mainly alewife (*Alosa pseudoharengus*) and round goby (*Neogobius melanostomus*; syn. *Apollonia melanostoma*) (Somers et al. 2003).

Compared to Lake Ontario, shallower, warmer Lake Erie supports larger populations of percids, notably walleye (*Sander vitreus*) and yellow perch (*Perca flavescens*) (Leach & Nepszy 1976). However, introduced species have also likely caused declines in native benthic

fish species, with round goby the most numerous and geographically widespread small benthic fish in recent surveys of northern Lake Erie (Reid & Mandrak 2008). These recent invasive changes in fish populations have likely further affected cormorant diet, which was already known to differ between Lakes Ontario and Erie (Bur et al. 1999; Johnson et al. 2010; Burnett et al. 2002).

In addition to site differences, sex-based or individual foraging differences might promote variation in cormorant diet and thus affect contaminant uptake. Robinson et al. (2009) used C and N stable isotopes to demonstrate that males from Lake Ontario foraged more in pelagic areas, and at a slightly lower trophic level than females. Males are approximately 14-16% heavier than females (Bedard et al. 1995, Robinson et al. 2008). This may allow males to dive longer and deeper (as reported for other species of cormorants, e.g. (Kato et al. 1999; Quintana et al. 2011)), and perhaps fly farther: for example, in foraging trips during the nesting season, males most often foraged at 20-25 km from the colony, while females flew shorter distances to forage, with the highest proportion 5-10 km from the colony (Anderson et al. 2004). However, not all studies have found this pattern: Guillaumet et al. (2011) noted high inter-individual variability in movement unrelated to sex, which may be related to individual foraging choices in that individuals may fly an extremely long distance one day then forage close to the colony the next. Several years after the round goby invasion, some cormorants fed on gobies (i.e. a new prey item) while others did not, which could suggest some degree of specialization (Somers et al. 2003).

Causing further complexity in the study of cormorant diet, the variety of analysis methods used can influence diet estimates. Regurgitated samples are easy to collect, as both

adults and chicks often vomit during disturbance. In contrast to pellets (regurgitated indigestible portions containing bones and otoliths (Somers et al. 2003)), regurgitated fish are often in reasonably good condition and therefore easier for non-specialists to identify. However, serious biases in data resulting from this type of collection have become apparent (e.g. Seefelt & Gillingham (2006); Bearhop et al. (2001)). Newer biochemical approaches such as fatty acid analysis can use a single tissue sample to provide data that integrate a longer period of foraging (Williams & Buck 2010; Hebert et al. 2006). However, all methods used to study bird diet, including fatty acid analysis, will be subject to some bias and uncertainty (Barrett et al. 2007), so combining traditional methods with biochemical approaches can allow for a more balanced interpretation of results (Bearhop et al. 2001).

Polymethylene interrupted (PMI) fatty acids (also known as as NMI (non-methylene interrupted) fatty acids) are unusual C20 and C22 modified fatty acids produced only in certain aquatic invertebrates (Mezek et al. 2009, 2011; Barnathan 2009). PMI fatty acids are especially high in invasive dreissenid mussels. Since cormorants feed on fish that eat these mussels, including round gobies, (Marentette et al. 2010, Somers et al. 2003), PMI fatty acids should correlate with cormorants' relative 'secondary' mussel intake and could therefore be a useful indicator for possible mussel-transferred contamination.

In this study, we compare and contrast cormorant diets during the nesting season from two colonies in Hamilton Harbour, western Lake Ontario and one colony in northeastern Lake Erie, since differing diets could have implications for contaminant transfer to cormorants. We anticipated that diets at the two colonies in Hamilton Harbour would be similar due to large, overlapping foraging ranges between these two areas. If so, cormorants at these two colonies

would presumably be receiving similar contamination through their diet, a question we wished to address for our concurrent study on mutation rates (Chapter 2). We analyzed cormorant diet using two approaches: item counts from stomach regurgitate, and fatty acid analyses (both 'regular', i.e. methylene-interrupted, and PMI fatty acids), predicting that these different measurements of diet will generate similar diet composition estimates.

Methods

Three similarly sized colonies of ground-nesting cormorants (each consisting of approximately 1000 breeding pairs) on the lower Great Lakes, Ontario, Canada were chosen for sampling, for a concurrent study on contaminants. We chose two colonies in Hamilton Harbour to compare, plus a reference site in cleaner Lake Erie. Colonies at Pier 27 and Farr Island, were located in Hamilton Harbour (43° 17' N, 79° 49' W), western Lake Ontario (Chapter 2, Figure 1; Farr Island was converted to underwater fish habitat in 2010; for descriptions of these sites see Somers et al. (2007) and Gebauer et al. (1992); for another detailed map including these two sites see Figure 1 in Quinn et al. (1996). Mohawk Island (42° 50' 2.5008" N, 79° 31' 22.497" W) is a National Wildlife Area in northeastern Lake Erie, near Rock Point at the mouth of the Grand River, approximately 2 km from shore (for a description of this site see King & de Solla (2010)).

Regurgitated diet samples

Regurgitated samples were collected from the ground (n = 55), adults (n = 27), chicks

of unknown sex ($n = 130$, 212 regurgitated masses in total) during cormorant breeding seasons between May and July 16 2009 and May 19 and July 13 2010. Of the 212 total regurgitated masses, 123 were collected from Pier 27, 64 from Farr Island, and 25 from Mohawk Island. Disturbance of cormorant colonies was necessary several times a day for our concurrent study, causing adults to regurgitate near their nest before flying away from the colony at our approach. We collected these fresh regurgitate samples from the ground of the colony, an approach used previously to study the diet of Double-crested Cormorants (e.g. Seefelt & Gillingham (2006)) and Great Cormorants (*P. carbo*), e.g. Lehikoinen (2005)). Regurgitate taken directly from adults was opportunistic (not purposefully induced) when they regurgitated during blood sampling or trapping (for a description of trapping methods, see Chapter 2). To sample regurgitations from chicks, we followed the procedures of Somers et al. (2003), using gentle abdominal stimulation while holding the bird tipped down such that its head was lower than its abdomen. When two chicks from the same nest were sampled, the regurgitate was pooled into a single sample (3 cases). Regurgitated samples were placed in labelled plastic bags and kept on ice during the field day, then frozen until analysis. Thawed regurgitated samples were weighed, and individual fish identified to the lowest taxonomic level possible, usually species. In cases where fish were too degraded to be identified with certainty, they were recorded as unknown. For statistical analysis, the three types of samples (from adults, chicks, and the ground) were pooled within each site to increase sample sizes and because we were attempting to define overall diet at the three colonies as opposed to partitioning it into chick and adult fractions; this pooling approach has been used previously in similar situations (e.g. Quillfeldt et al. (2011)). Differences among the three sites in numbers (counts) of round gobies, alewife, and cyprinid shiners were tested with chi square

contingency table analysis (other minor species that we found in very small percentages had to be excluded as they would cause chi square expected values to be too low, as in Somers et al. (2003)). We also then compared our alewife and round goby counts from our two Hamilton Harbour sites (Pier 27 and Farr Island) to those reported in Somers et al. (2003) from two Hamilton Harbour sites (including Farr Island) using a chi square test. Due to a lack of information on the sex of the cormorants contributing the regurgitated samples, and a lack of information regarding the time of day samples were collected, we did not analyze samples separately by sex nor time.

Fatty acid analysis

We trapped reproductive adult cormorants directly on their ground nests during peak breeding season between May and July in both 2009 and 2010. We used approximately 1 x 1.5 x 0.5 metre box traps made with chicken wire over a steel rod frame and a two-piece hinged wooden trigger connected to the back of the trap with clear monofilament line. Sampling and collection was approved by Environment Canada's Animal Care Committee and McMaster University's Animal Research Ethics Board (AUP # 08-06-31), samples collected under a Ontario Ministry of Natural Resources Wildlife Scientific Collector's Authorization (# 1051384), and cormorants banded under a Scientific Permit to Capture and Band Migratory Birds from the Canadian Wildlife Service (# 10529 AB).

Whole blood samples (up to 18 mL) were taken by brachial venipuncture from adult cormorants with 23 or 25 gauge Sarstedt Multifly butterfly catheter needles connected via Luer-Lock to either 6 or 12 mL Norm-ject syringes. All needles for blood sampling were rinsed

in 0.5 M EDTA immediately before use to prevent blood coagulation inside the needle.

Samples were stored on ice, then centrifuged for 6 minutes to separate plasma and cellular (largely red blood cell, or erythrocyte) fractions. These components were then separated and frozen in cryovials at -80°C until analysis. We chose red blood cells for analysis because they are easily obtained and have an appropriate turnover time as a medium-term storage component (Hobson & Clark 1993). We selected 15 samples from Pier 27, 14 samples from Farr Island, and 10 samples from Mohawk Island for a total of 39 samples for fatty acid analysis, choosing samples with the largest volumes to ensure they would be of adequate size for preparation and analysis. Cellular blood fractions were lipid-extracted and analyzed for fatty acids in the laboratory of Dr. Michael Arts, Environment Canada (for details, see Appendix 2).

Statistical analyses were performed in JMP 7.0 (SAS Institute Inc.). Fatty acids that were never detected ($n = 10$) or had a high proportion of non-detectable values ($n = 4$) in the cormorant samples were excluded from the analysis, such that from the 47 methylene-interrupted fatty acids originally measured, 33 were included in statistical analyses. Of these 33 fatty acids, six had a small proportion of values below the method detection limit (ranging from 2-14%, representing 1-5 missing values). Simplistic, albeit commonly used, methods for dealing with values below detection limits such as the use of one-half detection limit or the use of zeroes can obscure true trends in the data or create patterns where none would exist (Helsel 2006, de Solla et al. 2012). To resolve this problem, missing concentrations were imputed with a Maximum Likelihood Estimation (MLE) spreadsheet developed by Villanueva (2005) in Microsoft Excel with the iterative Solver function. This uses the individual detection

limit for each fatty acid to calculate replacement values which fit along a log-transformed quantile normal plot of the unbiased population mean and variance with maximum log-likelihood (Villanueva 2005). Values are then replaced sequentially according to their correlation with fatty acids that were detected in all samples, and each fatty acid then expressed as a percentage of the total fatty acids by weight for each individual. These percentages were then used for linear discriminant function analysis (DFA) (untransformed, using Wilks' Lambda adjusted to $p < 0.01$ due to non-normality of some fatty acids as in Quillfeldt et al. (2011)), and principal components analysis (PCA). Untransformed fatty acid data help to highlight differences between benthic and pelagic fish species (Czesny et al. 2011), which was important in our study since of the two main prey items round goby and alewife, one is benthic and the other pelagic (respectively). Mahalanobis distances were used to quantify differences among sites and compared with an ANOVA with Tukey-Kramer post-hoc test. Individual fatty acids and classes of fatty acids (e.g. n-6 fatty acids) were compared among sites with ANOVA and Tukey-Kramer HSD tests when normally distributed, and Wilcoxon/Kruskal-Wallis tests when non-normally distributed. Only one PMI fatty acid was reliably detected (of the 6 PMI fatty acids included), so concentrations and percentages (which were non-normally distributed) were compared among sites and sexes with Wilcoxon/Kruskal-Wallis tests.

Molecular Sexing

Adult cormorants used for fatty acid analysis were sexed using the procedure outlined in Fridolfsson & Ellegren (1999), in 10 μ L volumes with added bovine serum albumin (BSA). Products were separated and visualized on 1% agarose gels stained with ethidium bromide.

Results

Regurgitations

At Pier 27, Lake Ontario, out of a total count of 373 prey items from 123 regurgitated masses, 56.3% of prey items were alewife, 25.4% were round gobies, 13.4% were small Cyprinid shiners (likely emerald shiner, *Notropis atherinoides*), and 4.8% could not be identified with certainty. At Farr Island, Lake Ontario, out of a total count of 409 prey items from 64 regurgitated masses, 51.3% were alewife, 41.8% were round gobies, 3.9% were small Cyprinid shiners (likely emerald shiner), 0.7% were bluegill (*Lepomis macrochirus*), goldfish (*Carassius auratus*) and white perch (*Morone americana*) were 0.24% each, and 1.7% could not be identified with certainty. At Mohawk Island, Lake Erie, out of a total count of 151 prey items from 25 regurgitated masses, 70.1% of all items were round goby, 13.9% were small Cyprinid shiners (likely emerald shiner), 4.7% were yellow perch, 2.7% were smallmouth bass (*Micropterus dolomieu*), mudpuppy (*Necturus maculosus*) made up 2.6%, and 2% were alewife. Freshwater drum (*Aplodinotus grunniens*), white perch, and walleye each made up 0.66%, and lastly 2% could not be identified with certainty.

Differences in alewife, round goby, and Cyprinid shiners among the three sites were not significant ($p = 0.22, 0.19, \text{ and } 0.19$, respectively). Comparing our round gobies and alewife results from Pier 27 and Farr Island to those reported for two Hamilton Harbour sites in Somers et al. (2003), we found a significantly higher proportion of gobies and lower proportion of alewife ($\chi^2 = 139.14, \alpha = 0.05, p < 0.0001, df = 1, n = 1461$).

Regular fatty acids

The 'regular' (i.e. methylene-interrupted) fatty acids (Table 1) found in the highest percentages overall were palmitic acid (saturated, C16:0, 22.8%), followed by arachidonic acid (polyunsaturated, C20:4n6, 14.0%), then stearic acid (saturated, C18:0, 12.0%). The highest monounsaturated fatty acid, and fourth overall highest, was oleic acid (C18:1n9c, 9.3%). Percentages of n-6 fatty acids out of total regular fatty acids were higher at Farr Island and Pier 27, and significantly lower at Mohawk Island ($F = 9.81$, $df = 38$, $p = 0.0004$). Polyunsaturated fatty acids (PUFA) as a percentage of all fatty acids were higher in samples from Farr Island and Pier 27, and significantly lower at Mohawk Island ($F = 10.92$, $df = 38$, $p = 0.0002$).

The discriminant function analysis (DFA) correctly classified 100% of individual birds with respect to site (Wilks' Lambda = 0.0002, $p = 0.002$). Of the squared Mahalanobis distances, only Pier 27 to Mohawk and Farr to Mohawk were significant ($p < 0.0001$). The canonical biplot (Figure 1) showed Mohawk separated from both Pier and Farr along Canonical1 (the first canonical root), and Pier and Farr separately slightly along Canonical2, or the second canonical root. However, the first canonical root explained 97.9% of the variation in regular fatty acids, with the second canonical root explaining 2.09%. The DFA canonical biplot appears to show a large difference between the two Hamilton Harbour sites, but this can be misleading as that axis represents only 2%. Therefore, a principal components analysis (PCA, Figure 2) was also used to display the data. Principal Component 1 explained 39.1% of the variation, Principal Component 2 explained 23.0% of the variation, and Principal Component 3 explained 11.8% of the variation for a total of 74% of the variation explained by

the first three Principal Components. The PCA plot of PC1 against PC2 placed all the samples from Mohawk Island in one group; the remaining cluster contained a mix of both Pier 27 and Farr Island samples. A DFA correctly classified 100% of birds with respect to sex, but was not significant (Wilks' Lambda = 0.04, $p = 0.11$). The fatty acids on the canonical biplot that corresponded most strongly to females were C16:0i, C15:0, C:15ai. The PCA did not display any sex-specific differences in diet; male and female points were not distributed in any particular patterns on the plots.

PMI fatty acids

The only PMI fatty acid detected in every cormorant sample was C20:3n6 (Figure 3). The other five PMI fatty acids were detected at extremely small and varying concentrations in the 39 samples; C20:2n9 was detected in 22 samples (56%), C20:2n7 was detected in 6 (15%), C22:2n9 was detected in 27 (69%), C22:2n7 was detected in 21 (53%), and C22:3n6 was detected in 32 (82%). Due to these low detection frequencies, these five PMI fatty acids were not subject to further statistical analysis. Relative to total fatty acids, percentages of C20:3n6 from Mohawk Island, Lake Erie were approximately 5-6 and up to 43 times higher than those from Hamilton Harbour ($F = 74.29$, $p < 0.0001$, Figure 3). Concentrations of C20:3n6 ($\mu\text{g}/\text{mg}$ dry weight of tissue extracted) showed approximately the same relationship (data not shown). No sex differences in the concentrations nor percentages of C20:3n6 were found when all three sites were considered together ($F = 0.72$, $p = 0.39$; $F = 0.96$, $p = 0.33$), nor when analyzed separately by site (percentages: Pier 27 $F = 0.79$, $p = 0.38$, Farr $F = 2.74$, $p = 0.12$, Mohawk: $F = 0.02$, $p = 0.87$; concentrations: Pier 27 $F = 1.96$, $p = 0.18$, Farr $F = 2.7$, $p = 0.12$, Mohawk $F = 0.008$, $p = 0.93$).

Discussion

Double-crested Cormorants forage flexibly. Since the appearance of alewife in the Great Lakes, they have consistently been an important prey item for cormorants (Hatch & Weseloh 1999). Following the more recent invasion of the round goby to the Great Lakes around 2000, cormorants rapidly adapted their diets, with adults preying heavily on gobies (Johnson et al. 2010). Our regurgitate results supported these previous studies, in that diets were dominated by round goby and alewife. In eastern Lake Ontario, adult cormorant diets are now dominated by round goby (75-79% by frequency depending on the colony) in pre-chick, chick-feeding, and post-chick periods. Alewife was the second-most important species, consumed in the highest percentages (15-30%) during the chick-feeding periods (Johnson et al. 2010). In Hamilton Harbour, Somers et al. (2003) found 68-98% alewife and 0-21% round goby by frequency depending on the day sampled in chick regurgitate. We found round gobies at significantly higher overall frequencies in Hamilton Harbour, 25-42% by colony, suggesting their increasing importance since 2002. Alewife, which we found at 51-56% frequency by colony in Hamilton Harbour, showed a decrease from 2002 concurrent with the increase in gobies. Cyprinid shiners, also reported as a minor prey item in Hamilton Harbour (Somers et al. 2003) appeared important in diets at Pier 27, representing 13% of prey items, but small fish seem overrepresented when using frequency counts (since this assesses diet numerically rather than by weight, Lehtikoinen (2005)). At Mohawk Island, Lake Erie, diets were composed mostly of round goby (70%). Other species found at lower frequencies that were not identified in Hamilton Harbour diets were yellow perch, smallmouth bass, and

mudpuppies, which represented the only non-fish item identified.

Since cormorants are highly opportunistic feeders, prey usually reflects species availability in a given area (Hatch & Weseloh 1999), and can therefore can also serve to reflect relative numbers of most fish species, an important measure of ecosystem health. Our results from regurgitate identified the same main species found in surveys of fish communities in Hamilton Harbour. Alewife is the most abundant prey fish species in Lake Ontario (Hebert et al. 2008) and the most common offshore species in Hamilton Harbour, comprising up to 62% of total catch between the years of 2002-2008 (Brousseau & Randall 2008). Yellow perch may be 'virtually absent' in Hamilton Harbour; recent declines may be related to cormorant predation (reviewed in Brousseau & Randall (2008)), but Lake Erie supports larger populations of perch (Bur et al. 1999).

Regurgitate integrates only a short amount of time (Johnson et al. 2010). Important sources of bias can include the fact that adults usually only regurgitate food which was held in the proventriculus, some species are easier to regurgitate or predigest than others, and there can be differential digestion of the hard and soft portions of food (Barrett et al. 2007). Perhaps reflecting these digestion rate differences, Johnson et al. (2010) reported that at one site, alewife was the most common prey item in stomach samples, but round goby was the most common using pellets. This suggests that our regurgitate results could also be biased towards higher frequencies of alewife in the diet. However, we found good general concordance with the results from regurgitate compared to fatty acid analysis.

Choice of tissue type for fatty acid analysis

We chose to use the cellular fraction of blood since plasma might not integrate enough long-term information about diet, and would skew results towards recent consumption. Longer term measures such as analysis of adipose tissue are logistically difficult and might integrate diet information from wintering locations or migration, which would complicate interpretation. In isotope studies in birds, blood cells are thought to integrate up to two months of diet (Hobson & Clark 1993), so this timeline may be similar for fatty acid analysis. Red blood cells have an approximate life span of 28-35 days in chickens, 35-45 days in pigeons, 33-35 days in quails (Thrall et al. 2004) and 39-42 days in ducks (Campbell et al. 2010; Brace & Altland 1956). Based on this evidence, we estimate our samples were integrating diet information for approximately 1.5 months, such that since most samples were taken in late May and June, they could represent diet beginning from mid-April to mid-May, when the adults were at the colony nesting and feeding chicks. The time we examined covered mostly the chick-feeding period (though various nests in the colony are at different stages at any one time) such that the timing of fatty acid profiles correlates reasonably well with the periods during which we collected regurgitate samples in the colonies. A comparison of our two sets of results also demonstrated the sensitivity of fatty acid analysis compared to regurgitate, in that even with over 200 diet samples a difference between sites in prey items using regurgitate was not significant, but with fatty acids even the very minor differences between Pier 27 and Farr Island could be used to separate these two groups. This points to the clarity and resolution power of fatty acid analysis, even with a relatively small number of samples from each site.

Fatty Acid profiles

Cormorant samples showed high proportions of highly polyunsaturated n-3 fatty acids, representative of all fish-eating birds (Surai et al. 2001). Fatty acid profiles suggest adult diet differs greatly between Mohawk and Hamilton Harbour, but is very similar at the two Harbour sites (Pier 27 and Farr Island).

While fatty acid profiles are generally conserved from prey to predator, metabolism of some fatty acids means some will be better indicators of diet than others. In a captive feeding trial with Herring Gulls (*Larus argentatus*), Käkälä et al. (2009) demonstrated that certain fatty acids respond to changes in diet reliably, while others are probably modified enough by metabolism to represent little value in quantifying diet. Therefore, these individual fatty acids that responded to diet changes in Käkälä et al. (2009) may be among the best indicators of diet in cormorants as well. One useful fatty acid in their study was C18:1n-7, which was on average higher in cormorants from Mohawk Island, Lake Erie. This fatty acid originates in benthic microbes, and was plentiful in bottom-dwelling fish in their study. Concentrations in round gobies were among the highest in multiple fish species analyzed by Czesny et al. (2011). Regurgitate samples suggested diets at Mohawk are dominated by round goby, a bottom-dwelling species, so higher concentrations of C18:1n-7 in Mohawk samples supports data obtained from the regurgitate. The saturated fatty acid C14:0 was also found to agree closely with diet changes, since vertebrates do not produce it (Käkälä et al. 2009), indicating its presence in cormorants is completely due to dietary intake. C14:0 was higher at Farr Island and Pier 27, and lowest at Mohawk Island. C14:0 is derived from phytoplankton (Rossi et al. 2006) and approximately three times higher in alewife than round goby (Czesny et al. 2011). This suggests alewife is most important in the diet at Hamilton Harbour and less so at

Mohawk Island, agreeing well with regurgitate samples where we found a very low frequency of alewife in Mohawk samples and over 50% alewife in colonies at Hamilton Harbour.

C20:1n9 is a long chain polyunsaturated fatty acid derived from zooplankton (Käkelä et al. 2007) and a good indicator of diet (Käkelä et al. 2009). It is higher in alewife than round goby (Czesny et al. 2011), and approximately four times higher by percentage in cormorants from Hamilton Harbour, providing further support for the importance of alewife as a cormorant prey item at Hamilton Harbour but not Mohawk Island. C16:1n7 percentages were approximately five times higher at Mohawk Island than Pier or Farr. This fatty acid is found in relatively low percentages in alewife, medium percentages in round gobies, and high percentages in adult yellow perch and represents a benthic signature (Czesny et al. 2011), and so may reflect the near-absence of alewife in Mohawk diet samples, the importance of gobies, plus the taking of yellow perch, a prey item which was not recorded in Hamilton Harbour. Providing further support for yellow perch as a diet component, C17:0 percentages were much lower at Mohawk Island than Pier 27 or Farr Island, and adult yellow perch had the lowest C17:0 percentages in Czesny et al. (2011).

Several studies have used fatty acid analyses in various ways to assess diet in seabirds and waterbirds. Seasonal diet changes from squid to crustaceans were reflected in fatty acids of Thin-billed Prion (*Pachyptila belcheri*) regurgitate (Quillfeldt et al. 2011). Tierney et al. (2008) showed high variation in the diet of the Adélie penguin (*Pygoscelis adeliae*) related to age, year, and breeding stage. Double-crested Cormorant eggs from Saskatchewan, Canada contained high concentrations of fatty acids resulting from freshwater fish species, especially C20:4n-6 (Surai et al. 2001), as did eggs of the Little Cormorant (*P.*

niger, Ramírez et al. 2009), and we also found this fatty acid in similarly high proportions at all three sites. Käckelä et al. (2007) used plasma from shags (*P. aristotelis*) to demonstrate diets with low interindividual variability dominated by pelagic fish species. Our fatty acid profiles also seem to show little interindividual variability in diet, judging by the small amounts of variability within colonies overshadowed by large differences among lakes. This suggests that rather than specializing on preferred species, cormorants forage opportunistically on the species that are the most abundant, easiest to catch, or both, as is probably the case for alewife and round gobies.

PMI fatty acids and mussel-transferred contamination

Cormorants at Mohawk Island, Lake Erie had relatively high concentrations and percentages of the PMI fatty acid C20:3n6. In zebra and quagga mussels (*Dreissena* spp.) from Lake Ontario, PMI fatty acids make up 4-6% of total fatty acids (Mezek et al. 2011). PMI fatty acids are transferred and stored in organisms that consume mussels, and in species that then secondarily consume mussels. Therefore, it is possible to trace mussels upwards through aquatic food webs, and PMI fatty acids have been detected in seals, walruses, (Budge et al. 2007) and polar bears (Thiemann et al. 2007). Käckelä et al. (2007) reported one PMI fatty acid in shags (*P. aristotelis*) at a mean molar percentage of 0.08 (20:2NMID), representing 20:2n-7 and 20:2n-9. These PMI fatty acids were detected in some samples in our study, but had a very high percentage of non-detected values. This is probably a reflection of the different relative amounts of individual PMI fatty acids available in these two very different food webs (Atlantic Ocean for shags, Great Lakes for cormorants). Higher percentages of C20:3n6 in samples from Mohawk Island suggest cormorants at this site

secondarily consume much higher proportions of mussels. This is likely because round gobies in Lake Erie consume almost entirely mussels, 92% mussels by frequency in Andraso et al. (2011). In contrast, round gobies consume mussels in small proportions at study sites within Hamilton Harbour: chironomid insect larvae were the most common prey item, and mussels were found in only 16% of stomachs (Empringham 2011). In eastern Lake Ontario, Johnson et al. (2008) also found chironomids to be the major prey item in round goby diets during all times of day, at up to 71% by dry weight. Mussels are probably less available to round gobies (and other fish species) in Hamilton Harbour because the density of dreissenid mussels is much lower compared to Lake Erie (Dermott et al. 2007).

Round gobies can transfer contaminants and metabolites of contaminants to their predators in these lake ecosystems (Levengood & Schaeffer 2011; Kwon et al. 2006). This is largely due to round gobies specializing on mussels, which bioaccumulate pollutants such as hydrocarbons and heavy metals as they lack adequate cytochrome P450 and advanced Ah receptors (Hahn et al. 1994, Hahn 2002). By feeding on other, small food sources, gobies in Hamilton Harbour may be limiting their body burdens. However, alewife from areas with contaminated sediment can also have high body burdens of PAHs (Levengood & Schaeffer 2011), even though it is a planktivorous species. At Mohawk Island, diets are comparatively high in gobies which could potentially lead to a more toxic diet for those cormorants, if the gobies consumed contaminated mussels. However, this is not likely since northeastern Lake Erie sediments are much less contaminated than those from Hamilton Harbour (Marvin et al. 2002). Mudpuppies, found in the diet at Mohawk Island but not Hamilton Harbour, may

consume fish species that eat mussels, or even the mussels themselves (A. Gendron, pers. comm.; Gendron et al. (1999)), so they may also be also acting as an important conduit for PMI fatty acids to cormorants. Additionally, mudpuppies consume various other molluscs, mostly gastropods (Gendron et al. 1999) which may produce PMI fatty acids as well (Barnathan 2009), although the specific species present at study sites have never been analyzed.

PMI fatty acids were not different among the two Hamilton Harbour sites, and overall, the two Hamilton Harbour populations showed very small differences in diet with either regurgitate or fatty acids. Differential sedimentary contamination in areas where the cormorants from the two colonies are feeding could still promote contamination differences in diet, but this seems improbable since cormorants forage at long distances from their colonies (Ridgway et al. 2006; Ridgway & Middel 2011) so the two colonies in Hamilton Harbour are likely to be using the same foraging range. Therefore, differences in exposure between the two sites seems less likely to result from variation in prey species consumed and more likely to result from non-dietary routes of exposure, such as the airborne particulate present in Hamilton Harbour (Sofowote et al. 2008).

Sex differences in diet

Some fatty acids showed minor sex-based differences in diet. The fatty acids that best separated females from males included those originating in bacteria, both benthic and intestinal (Käkelä et al. 2009); this may suggest higher consumption of gobies in females, or alternately different gut microbial compositions between the sexes. Fenech et al. (2004)

reported that in Arkansas, male and female diets varied in composition with respect to both forage fish and sport fish species. On the Columbia River between Oregon and Washington, males used a wider variety of foraging techniques, and took prey in benthic areas more often (Anderson et al. 2004). However, Guillaumet et al. (2011) found no difference in the home range sizes of males and females from multiple colonies across four Great Lakes fitted with satellite transmitters. Stable isotope ratios have suggested males from Lake Ontario foraged at a slightly lower trophic level than females (Robinson et al. 2009). In contrast, in Imperial Shags (*P. atriceps*) where males were 27% heavier, males foraged at a higher trophic level thought to be related to their larger size. However, they did not dive deeper or make longer foraging trips every year (Quillfeldt et al. 2011). Overall, these studies suggest foraging patterns are not consistent across multiple areas, time periods, sexes, or colonies, but rather are highly flexible.

Invasive species have clearly caused great change in cormorant diets, and previous conclusions made before the arrival of round gobies are now being continuously re-evaluated (e.g. Somers et al. (2003); Johnson et al. (2010)). The arrival of the round goby and its preferred food source dreissenid mussels completes an “efficient contaminant-transfer pathway” to higher trophic levels (Kwon et al. 2006), demonstrating that these species, important in the cormorant food web, have both ecological and contaminant impacts. The round goby invaded Lake Erie before it was detected in Hamilton Harbour (Walsh et al. 2007; Johnson et al. 2005). The high frequency of round gobies in the Lake Erie diet may reflect a goby population that has had a longer time to grow, and in the future we may see cormorants in Hamilton Harbour eating higher proportions of gobies as well. This could increase

contaminants through diet if round gobies in Hamilton Harbour are more contaminated than alewives. Alternately, if increasing numbers of predators exploit round gobies and reduce their populations, the proportion of gobies in cormorant diets may begin to decrease, and a shift towards other, potentially less contaminated fish species could be observed. Goby populations may have been exhibiting a period of rapid initial growth during the first years of their colonization, so their percentage in cormorant diet may have been dramatically higher during that time (Johnson et al. 2010). However, the incidence of gobies has continued to increase in yearly electrofishing transects in the Harbour since their discovery (Brousseau & Randall 2008). Both round gobies and alewives can accumulate contaminants through diet, but whether changes in diet have direct consequences for cormorants is still unknown. Cormorants may be an important final spot for many contaminants present in Great Lakes food webs, regardless of which dietary pathways they follow. Continual study of cormorant diet using a variety of traditional and biochemicals methods, preferably in combination, will help to track the diet of this important Great Lakes predator in a changing ecosystem.

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Table 1. Fatty acid profiles from Double-crested Cormorant (*Phalacrocorax auritus*) red blood cell samples from three colonies in Ontario, Canada. Values (w/w percentages) are means \pm SD from n = 39 samples (15 samples from Pier 27, 14 samples from Farr Island, 10 samples from Mohawk Island). Not shown are the ten fatty acids that were not detected in any of the samples, nor the samples which showed a high proportion of non-detectable values and excluded from analysis. Asterisks indicate fatty acids which show significant differences among sites. Abbreviations i and ai after the fatty acid name stand for iso- and anteiso- (carbon branches) respectively; c and t indicate cis and trans respectively.

	Pier 27	Farr Island	Mohawk Island	Overall
	Lake Ontario	Lake Ontario	Lake Erie	
Saturated Fatty Acids (SAFA)				
C14:0 *	1.49 \pm 0.08	1.60 \pm 0.08	1.22 \pm 0.10	1.46 \pm 0.34
C15:0i *	0.22 \pm 0.05	0.27 \pm 0.06	0.17 \pm 0.05	0.23 \pm 0.06
C15:0ai	0.13 \pm 0.04	0.10 \pm 0.03	0.12 \pm 0.05	0.11 \pm 0.04
C15:0 *	0.44 \pm 0.08	0.42 \pm 0.06	0.30 \pm 0.03	0.39 \pm 0.08
C16:0i *	0.20 \pm 0.01	0.13 \pm 0.10	0.33 \pm 0.07	0.21 \pm 0.10
C16:0	23.06 \pm 1.36	22.58 \pm 1.63	22.85 \pm 2.01	22.83 \pm 1.61
C17:0 *	0.62 \pm 0.07	0.61 \pm 0.05	0.36 \pm 0.04	0.55 \pm 0.12
C18:0	11.67 \pm 1.19	11.82 \pm 1.82	12.85 \pm 1.64	12.03 \pm 1.59
C20:0 *	0.51 \pm 0.08	0.39 \pm 0.07	0.48 \pm 0.13	0.46 \pm 0.11
C22:0 *	0.47 \pm 0.13	0.32 \pm 0.08	0.38 \pm 0.25	0.39 \pm 0.17
C24:0	0.30 \pm 0.13	0.24 \pm 0.09	0.19 \pm 0.08	0.25 \pm 0.11
Monounsaturated Fatty Acids (MUFA)				
C15:1 *	0.16 \pm 0.04	0.13 \pm 0.06	0.23 \pm 0.13	0.17 \pm 0.09
C16:1n7 *	1.39 \pm 0.29	1.67 \pm 0.37	5.46 \pm 2.46	2.53 \pm 2.13
C18:1n9t *	0.19 \pm 0.05	0.18 \pm 0.02	0.43 \pm 0.11	0.25 \pm 0.12
C18:1n9c *	9.61 \pm 2.20	10.51 \pm 2.28	7.23 \pm 1.16	9.32 \pm 2.36
C18:1n7 *	6.65 \pm 0.93	6.27 \pm 0.59	8.33 \pm 0.92	6.94 \pm 1.16
C20:1n9 *	1.20 \pm 0.29	1.06 \pm 0.12	0.33 \pm 0.08	0.93 \pm 0.41
C20:1n7 *	0.13 \pm 0.03	0.14 \pm 0.02	0.21 \pm 0.08	0.15 \pm 0.05
C22:1n9 *	0.27 \pm 0.07	0.22 \pm 0.02	0.09 \pm 0.04	0.21 \pm 0.09
C24:1n9 *	0.93 \pm 0.26	0.74 \pm 0.10	0.33 \pm 0.09	0.71 \pm 0.29
Polyunsaturated Fatty Acids (PUFA)				

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C18:2n6c *	3.74 ±0.57	3.45 ±0.18	1.71 ±0.23	3.12 ±0.92
C20:3n6 *	0.47 ±0.08	0.41 ±0.02	0.24 ±0.03	0.39 ±0.10
C18:3n6 *	0.13 ±0.02	0.15 ±0.02	0.10 ±0.02	0.13 ±0.03
C18:3n3 *	1.13 ±0.28	1.28 ±0.34	0.78 ±0.30	1.09 ±0.36
C18:4n3	0.50 ±0.13	0.59 ±0.20	0.66 ±0.47	0.57 ±0.28
C20:3n3 *	0.67 ±0.07	0.66 ±0.06	0.22 ±0.07	0.55 ±0.20
C20:4n6	13.8 ±1.60	14.65 ±1.24	13.46 ±2.09	14.05 ±1.66
C22:2n6 *	0.18 ±0.04	0.17 ±0.02	0.05 ±0.04	0.14 ±0.06
C20:2n6 *	0.86 ±0.13	0.78 ±0.04	0.28 ±0.07	0.69 ±0.26
C20:5n3	5.42 ±1.25	5.59 ±1.29	5.68 ±1.59	5.55 ±1.32
C22:4n6	1.97 ±0.32	1.90 ±0.23	2.10 ±0.53	1.98 ±0.36
C22:5n3c *	3.02 ±0.31	2.93 ±0.45	4.46 ±0.52	3.36 ±0.77
C22:6n3	8.18 ±0.81	7.67 ±0.85	8.01 ±1.38	7.95 ±1.00
Total n-3	18.95	18.76	19.83	19.1
Total n-6 *	20.200	20.6	17.64	19.68
n-3/n-6 ratio	0.930	0.91	1.12	0.97
Total SAFA	39.22	38.59	39.41	39.04
Total MUFA	20.57	20.98	22.76	21.28
Total PUFA *	40.2	40.42	37.82	39.67

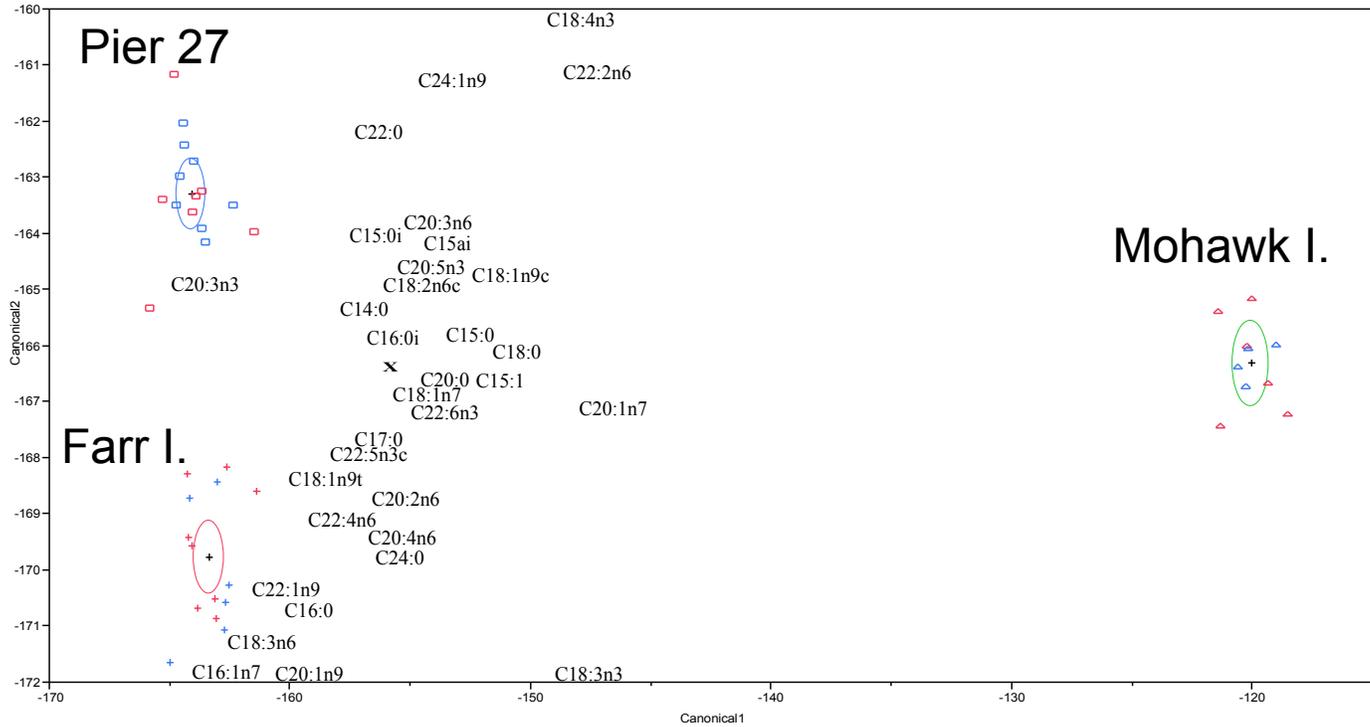


Figure 1. Canonical plot from linear discriminant function analysis on proportions (percentages) of 33 methylene-interrupted fatty acids. Ellipses represent the 95% confidence region estimated to contain the true mean of each site. Samples from females are coloured pink and those from males blue. A total of 39 samples are represented (14 from Farr, 10 from Mohawk, 15 from Pier). There were no misclassifications among the three groups. The black x represents the origin of the removed biplot rays. Fatty acid label names (e.g. C18:3n3) represent the end location of biplot rays, which indicate direction of relationship. Since they indicate general direction not position, movement does not affect interpretation and some fatty acid labels were moved slightly for clarity (i.e. to avoid overlapping text). Mahalanobis distances: Pier 27 to Pier 27 = 30.57, Farr I. to Farr I. = 30.12, Mohawk I. to Mohawk I. = 30.76, Pier 27 to Farr I. = 73.04, Pier 27 to Mohawk I. = 1979.40, Farr I. to Mohawk I. = 1916.94.

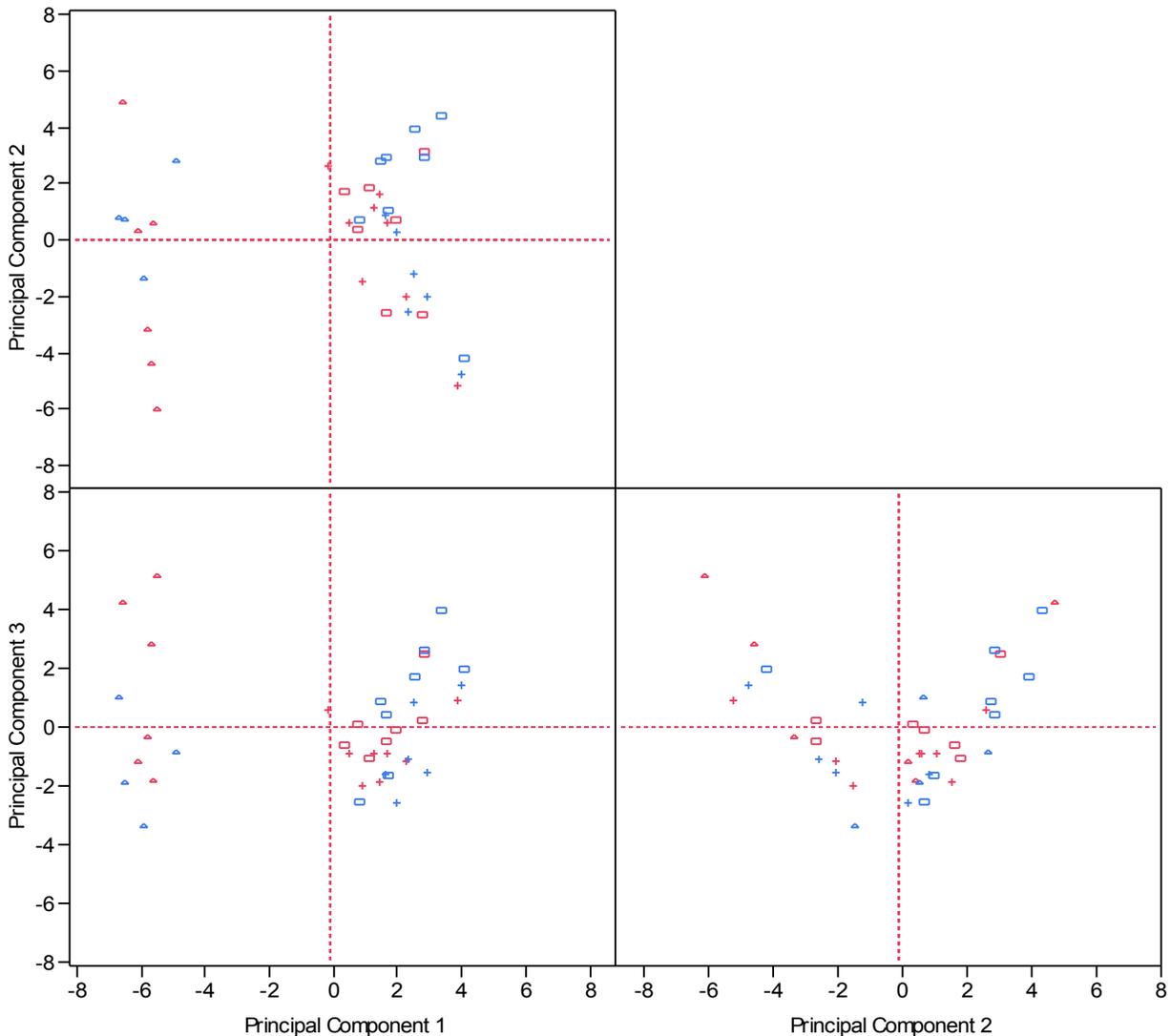


Figure 2. Principal components analysis (PCA) on 33 'regular' (i.e. methylene-interrupted) fatty acid percentages of total fatty acids analyzed from the cellular blood fraction in adult cormorants. Principal Component 1 explains 39.1% of the variation, Principal Component 2 explains 23.0% of the variation, and Principal Component 3 explains 11.8% of the variation for a total of 74% of the variation explained by the first three Principal Components. Samples from Pier 27 are indicated with a rectangle, samples from Farr Island are indicated with a cross, and samples from Mohawk are indicated with a triangle. Samples representing females are coloured pink and those representing males are blue.

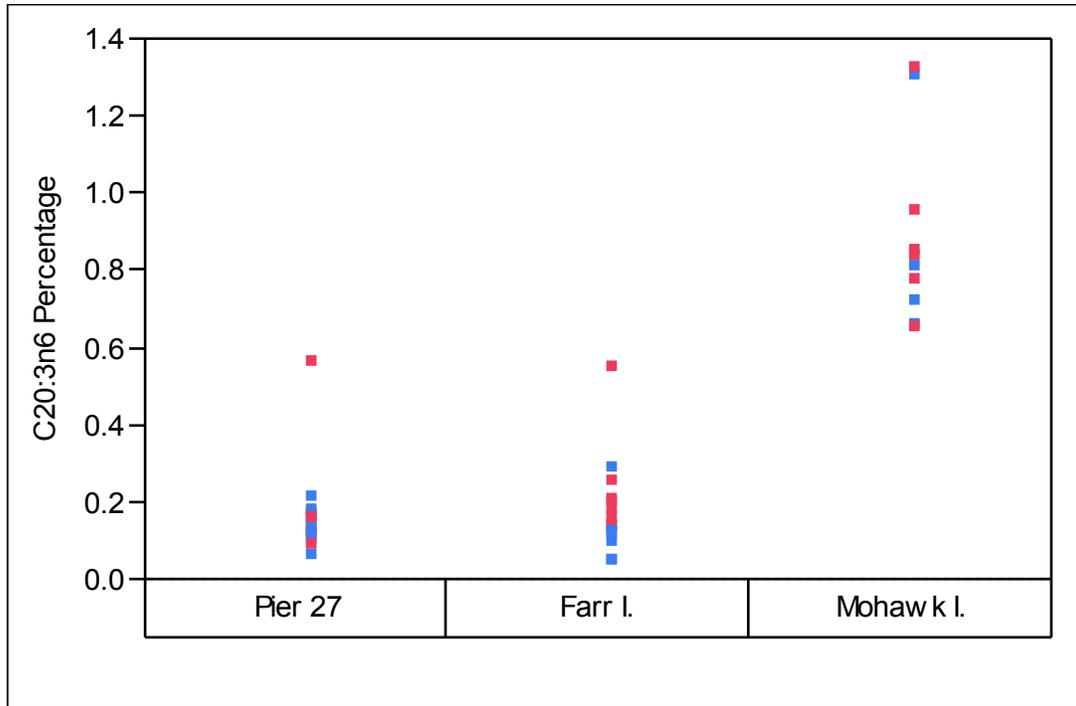


Figure 3. Percentages of C20:3n-6, a polymethylene-interrupted (PMI) fatty acid in samples of cormorant red blood cells from three sites in Ontario: Pier 27 and Farr Island, Hamilton Harbour, Lake Ontario, and Mohawk Island, Lake Erie. In total 39 samples are represented (15 from Pier 27, 14 from Farr, 10 from Mohawk). Percentage is calculated as concentrations of C20:3n6 divided by total concentration (total = 33 regular fatty acids used for analysis plus this one PMI fatty acid used for analysis). Samples from females are coloured pink and those from males are blue.

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Chapter Four: General Conclusion

In this thesis, I set out to address whether proximity to industrial air pollution was correlated with microsatellite mutations in families of cormorants, and if diet had the potential to have caused these changes. Germline microsatellite mutations had not previously been demonstrated due to environmental contamination other than radiation. This thesis also contributes towards the call for “Germline mutation studies in additional wildlife species” put forward by Somers (2004).

Chapter 2 describes an increase in mutations at the site nearest to the Hamilton Harbour industrial area (Pier 27), compared to another site in Hamilton Harbour (Farr Island) and a cleaner reference site (Mohawk Island). This chapter also demonstrated a metabolite of the PAH benzo[a]pyrene in cormorant chick liver and bile, showing that cormorants in Hamilton Harbour are processing this PAH through their body tissues. The complicated nature of environmental exposure in this contaminated area, with multiple exposure routes, means that cormorants in Hamilton Harbour likely absorb genotoxins daily from contaminated soil and water, breathe contaminated air, and feed on a variety of contaminated aquatic species. Teasing apart the relative contribution from each source presents a substantial challenge which can often only be addressed with laboratory or relatively controlled caged-animal studies (e.g. Somers et al. (2004)). However, we chose the two Hamilton Harbour colonies (Pier 27 and Farr Island) on the basis that they likely have similar diets and traffic contaminant exposure, yet differences in exposure to industrial air pollution, Pier 27 being located closest to the steel mills. In this way, the impact of the one variable 'distance from the industrial core'

on mutation rates could be tested since these populations have only small differences in diet composition, but marked differences in industrial air pollution exposure. Potential contribution of diet was then examined in Chapter 3, where we showed that cormorant diet at the two Hamilton Harbour sites was very similar. This illustrates the low likelihood that diet could have been a major contributor to the differences in induced mutations between the two sites. In comparing the relative mutation rates in the two Hamilton Harbour sites, the much higher mutation rate at Pier 27 demonstrated that differences in contaminant uptake via diet differences are likely not responsible for any observed patterns in the incidence of mutations, but rather are related to airborne PAH exposure.

Studies of wild, free-ranging animals for genotoxicity

Toxicological research on wild animals has “inherent value in providing a real-world signal of contaminant transport and fate” but can lack “mechanistic certainty” (Harris et al. 2011). This type of trade-off was apparent in this study, where cormorants were exposed to a whole contaminated environment and lived normally, without experimental interference. However, as discussed above, the variety of exposure routes and times in an environmental setting can complicate interpretation. For example, parental pre-meiotic and post-meiotic exposures can influence mutations in offspring in different ways (Yauk & Polyzos 2005) but since cormorants were constantly living in a contaminated environment they were exposed at both stages. Exposure times and types probably interact with mutation mechanisms in both somatic and germline DNA in ways we do not understand, and will be an interesting direction to pursue in laboratory research.

Previous microsatellite mutation research in Hamilton Harbour revealed no significant increases in mutation compared to reference groups. Comparisons with these studies of Hamilton Harbour sediment-exposed fathead minnows (Miller 2009) and wild tree swallows (Anmarkrud et al. 2011) may serve to underline important differences contributing to our detection of significant effects in cormorants. As discussed in Chapter One (Introduction), the body size of cormorants is much larger relative to these two previously used species, and relative capacities to repair DNA damage and avoid mutations could be unique to each species. For example, fish from contaminated areas have displayed some resistance to PAH-induced toxicity, often by increasing detoxifying enzymes (Wirgin & Waldman 2004), a phenomenon that has not been reported in birds. Additionally, it is possible that the aquatic nature of the minnow exposures produced different or less severe mutation effects, or that the commercial frozen brine shrimp fed to minnows was less contaminated than natural Hamilton Harbour food sources (Miller 2009). The tree swallow breeding site was not directly downwind of the industrial emissions as is the cormorant colony at Pier 27. However, given that both studies did show a trend towards more mutations in the exposed groups, a more relevant point may be characteristics of microsatellite markers used in each study. The observed heterozygosity of the four markers screened in minnows were 0.75, two with 0.84, and 0.90, whereas in this study they were higher at 0.76, 0.86, 0.89, and two with 0.92 (Chapter 2, Table 1). Anmarkrud et al. (2011) used the highly variable marker HrU10 (observed heterozygosity 0.82 but 117 alleles) for identification of microsatellite mutations, but no additional loci; the five other loci also genotyped were simply used for parentage confirmation and not scored for mutations. In this respect, our use of five microsatellite loci that all had at least one mutation likely greatly increased power. In addition to the number of mutations

identified, which can be increased through the use of more loci, it is also relevant that even with high heterozygosity and number of alleles, some loci may not mutate at high rates for unknown reasons, so extra loci included in a study act as 'insurance' against this problem. Some studies have suggested perfect loci to be more mutable, and in this study we found the highest mutation rates in two perfect microsatellites (COR 03 and COR 06). The only locus to show mutations in Miller (2009) was a compound repeat $((CTAT)_{11}(CTGT)_{15})$, which could have caused its mutability to be lower than might be expected for a similar but perfect repeat locus.

Lastly, sample size and number of meioses scored is another important consideration, and one where this study exceeded those done previously. The sample size for mutation screening in Miller (2009) was 1080 offspring in 50 families and just 160 offspring in 53 families for Anmarkrud et al. (2011). Since these studies scored mutations at just one locus the numbers of meioses scored were respectively 2160 (1080 x 2) and 320 (160 x 2), whereas in this study it was 2480 (248 chicks x 10 alleles each). Clearly, the tree swallow study used a very small sample size comparatively, but the number of meioses scored in minnows approached that of this study. However, the additional consideration of 50 minnow families versus the 97 cormorant families included here shows the number of families was almost double in this study, and since statistical power is strongly dependent not on offspring per se but on the number of families (since offspring are related), this is another important factor that may have contributed towards our significant finding.

Airborne PAHs in Hamilton Harbour and other locations

Airborne PAH concentrations at the monitoring station near Pier 27 were relatively high during this study, exceeding provincial health guidelines, and total PAHs of the 8 PAHs measured ranged from approximately 1 – 58 ng/m³ during 2009 and 2010 (Chapter 2). Since studies almost always choose different PAHs to measure, a direct comparison of this 8-PAH total is not possible, but individual PAHs can provide a useful measure. Across 22 countries and 71 air samplers in Europe, benzo[a]anthracene concentrations never exceeded 0.6 ng/m³ (Jaward et al. 2004), while in Hamilton Harbour even the median was higher in both study years (0.74, 0.75) and the mean was much higher (1.05, 1.79). The same approximate patterns held true for chrysene (maximum 1.3 ng/m³ in Europe, means 1.69 and 2.22 ng/m³ in Hamilton Harbour), benzo[a]pyrene (maximum 0.25 ng/m³ in Europe, means 0.98 and 1.73 ng/m³ in Hamilton Harbour), indeno[1,2,3-cd]pyrene (maximum 0.13 ng/m³ in Europe, means 1.21 and 1.59 in Hamilton Harbour), and benzo[ghi]perylene (maximum 0.28 ng/m³ in Europe, means 1.00 and 1.54 ng/m³ in Hamilton Harbour, all European figures from Jaward et al. (2004)). Relative to Toronto, Ontario, Hamilton Harbour airborne PAH concentrations were much higher for all 8 PAHs, up to 36 times higher concentrations for some PAHs (e.g. in ng/m³: benzo[a]anthracene: up to 0.21 in Toronto, up to 9.6 in Hamilton; benzo[a]pyrene: up to 0.18 in Toronto, 8.1 in Hamilton; chrysene: up to 0.32 in Toronto, 10.3 in Hamilton; dibenzo[a,h]anthracene: up to 0.02 in Toronto, 1.6 in Hamilton (Motelay-Massei et al. 2005)). These monitors in Toronto included downtown urban areas, but these areas are without the major integrated steel industry found in Hamilton Harbour. Methodologies varied between these studies, but the very large magnitude of these differences where means in Hamilton Harbour were more than an order of magnitude higher than even the maximum reported elsewhere serves to illustrate the high degree of contamination found at the Hamilton Harbour

site.

Comparisons with Asia demonstrate an entirely different pattern. Benzo[a]anthracene concentrations ranged from 0.4 to 31.5 ng/m³ across five countries (Chang et al. 2006), while in Hamilton Harbour they were much lower, from 0.08 to 9.6 ng/m³ during the two study years. Chrysene concentrations were 0.34 to 3.62 ng/m³ in Asia, and from 0.22 to 10.3 ng/m³ in Hamilton Harbour, but chrysene was not measured at the most contaminated Asian sites. Benzo[b]fluoranthene reached a high of 154 ng/m³ in Asia, but never exceeded 10.3 ng/m³ in Hamilton Harbour. Patterns were similar for other PAHs such as benzo[ghi]perylene, indeno[1,2,3-cd]pyrene, and others, with Asian concentrations regularly exceeding those in Hamilton Harbour (all Asian values from (Chang et al. 2006)). These results demonstrate that the concentrations of PAHs that cormorants were exposed to during their breeding seasons in 2009 and 2010 are probably well above concentrations in other parts of Ontario and Europe, but lower than those found in various countries in Asia. While Hamilton Harbour is home to the two largest steel mills in Canada (City of Hamilton 2006), Canada is still a minor steel producer globally (16th worldwide, Hindle & Dehlen (2008)), and the much larger complexes in countries such as China, South Korea, India, and Russia are of global environmental concern. In Hamilton Harbour, steel mills represent a major source of PAHs, but additional important sources of PAHs in other areas include coal-fired generating stations, traffic, and other industrial facilities.

Sequence-based versus epigenetic changes in genotoxicology

While tandem repeat DNA has been a crucial tool for mutation detection in recent years, the implementation of epigenetic biomarkers in addition to sequence-based tools will

strengthen conclusions. Microsatellites will probably remain useful for several more years (Ellis et al. 2011; Moran et al. 2006), including for mutation detection, but the variety of new genomic technologies available are already providing interesting and relevant ways to examine damage due to chemical exposure. Epigenetic mechanisms are more important than sequence changes in many types of cancer (Feinberg et al. 2006), so the importance of extending research in this direction cannot be overstated. Hamilton Harbour air pollution can induce aberrant methylation patterns (global hypermethylation in sperm DNA) in mice (Yauk et al. 2008) and it is quite possible that cormorants from Pier 27 would have shown similar epigenetic changes. Exposure to air pollution and components such as PAHs and benzene can produce DNA methylation changes in humans as well (Bollati et al. 2007; Baccarelli et al. 2009; Perera et al. 2009). Transposons can be activated by chemical genotoxins including PAHs *in vitro* (mouse cells: Lu et al. (2000), human cells: Stribinskis & Ramos (2006)). This raises the possibility that mutations found in wildlife or humans could be partially due to mobile genomic elements that have been 'turned on' by pollutants (Mansour 2007; Stribinskis & Ramos 2006). These types of results point towards a more dynamic model of DNA: one in which epigenetic modifications are constantly changing in response to stressors including chemical exposures. If this sort of model proves true, we will need to rethink our understanding of chemically-induced DNA damage, especially as it relates to health effects, to include these important non-sequence-based changes. The dichotomy between germline and somatic DNA damage may blur, as chemically induced epigenetic changes also have the potential to affect both the organism and its offspring.

Linking mutations to health effects and study extrapolation

The health implications of the type of mutations identified in this study are unclear. The microsatellite mutations presented in this study may or may not be associated with current or future phenotypic effects (such as disease) in these cormorants, or effects at the population level. While certain microsatellite mutations can cause disease (see Chapter One (Introduction), *Induced Mutations*), the health effects of a higher microsatellite mutation rate or its correlates are unknown. PAHs adversely affect birds in a variety of ways, including reduced immune system function, egg production, and hatching success, and impaired liver function (Albers 2006), but whether these effects would be correlated with increased microsatellite mutation rates is not certain. Many species do have mechanisms to compensate when living in contaminated areas. For example, ring-billed gulls (*Larus delawarensis*) in Hamilton Harbour did not show more lung pathologies when compared to those collected in rural areas, which could suggest (among other explanations) some degree of resistance (Yauk et al. 2001). The connection between mutations in tandem repeat DNA and health effects is an important area for researchers to explore in the coming years. Correlations and causations between genomic instability, and development of disease such as cancer should be evaluated, along with demonstrable effects on wildlife populations like reproductive success and susceptibility to disease. The utility of tandem repeat DNA as a biomarker will be greatly increased when these types of relationships are better understood.

Understandably, there is a desire to understand the implications of this type of study to human health, but there are many reasons that extrapolation of results in birds or other animals to effects in humans is difficult. Biochemical activation of PAHs is required for most toxic effects, and so the relative amounts of various activating/detoxifying enzymes is

important, but these ratios vary between species (Jacob 1996). As vertebrates, cormorants and humans share the P450 1A hepatic cytochrome family, but beyond this basic similarity there is a lack of detailed comparative information that could help extrapolations. Canaries were the original air pollution monitors in coal mines, and birds “continue to be indicators of contaminant stress...in the environment” (Foster & Wang 2011), but they may be actually more sensitive environmental indicators than are humans, due to very different lung structures and function (see Chapter One, *Cormorants as biological indicators of pollution*). However, there is no lack of evidence that airborne pollution containing PAHs does harm humans, including causing DNA damage (e.g. Pavanello et al. 2010; Okona-Mensah et al. 2005; Valavanidis et al. 2008; Laumbach & Kipen 2010; Besaratinia & Pfeifer 2008; Perera et al. 2005; Perera et al. 2009).

Conclusions

Humans and wildlife are threatened by pollution, being regularly exposed to a mixture of mutagenic and genotoxic compounds in their environments. Chemicals have recently been implicated as being critically important in explaining the increases in human cancer seen all over the world (Belpomme et al. 2007). Various chemicals in airborne pollution including PAHs can induce a variety of types of DNA damage, including germline microsatellite mutations in cormorants. Considerations of DNA-damaging effects of chemicals should not be limited to sequence changes, as epigenetic effects are likely incredibly important in regulating genes and genetic disease. Future research should work towards improving our understanding of DNA damage mechanisms, and use realistic studies replicating conditions closest to those currently faced by wildlife and humans.

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Appendix 1: Unknown LC-MS/MS peaks

Table A1. Unknown peaks at the same m/z but different retention time as 2-hydroxynaphthalene detected in cormorant fecal samples by LC-MS/MS.

Sample ID	Age	Sample Type	Site	Concentration in sample vial (ng/mL)	Concentration (ng/g) wet weight	Concentration (ng/g) dry weight
4	adult	fecal	Mohawk	2.09	4.14	20.49
9	adult	fecal	Mohawk	7.28	23.55	177.56
10	chick	fecal	Farr Island	8.02	13.01	41.77
11	chick	fecal	Mohawk	1.01	4.37	12.16
13	chick	fecal	Pier 27	4.12	8.76	91.55
17	chick	fecal	Farr Island	1.75	13.88	18.42
21	chick	bile	Farr Island	0.26	6.66	n/a
24	chick	bile	Farr Island	1.65	8.82	n/a
32	chick	bile	Pier 27	unable to quantify		n/a
35	chick	bile	Pier 27	0.86	14.83	n/a

Appendix 2: Details of fatty acid analysis

Performed in the laboratory of Dr. Michael T. Arts, Environment Canada, Canada Centre for Inland Waters, Burlington, Ontario, by Ashley Kling and Jerry Chao.

Cellular blood fractions were freeze-dried (approximately 30 mg for regular fatty acid analysis, approximately 275 mg for PMI fatty acid analysis) then lipids were extracted three times in 2:1 chloroform: methanol (Folch et al. 1957) with 5 alpha-cholestane added as an internal standard. Extracts were subsequently dried under a stream of nitrogen and stored at -85°C until the next step of analyses.

Fatty acids were methylated to fatty acid methyl esters (FAME) using the sulphuric acid in methanol method (Christie 2003). Regular fatty acids were detected with GC-FID (gas chromatography – flame ionization detector) in splitless mode, with a detector temperature of 250°C . Six PMI fatty acids were analyzed by GC-MS triple-quadrupole (Agilent 7890A GC with Agilent 7000 MS detector) in SCAN mode with a detector transfer line temperature of 250°C , helium at 2.25 mL/min as collision cell quench gas, and nitrogen at 1.5mL/min as collision gas. The inlet temperature was 250°C , and the initial oven temperature was 70°C . After 1 minute, the oven temperature ramped to 140°C , at a rate of $20^{\circ}\text{C}/\text{min}$, then 170°C at a rate of $4^{\circ}\text{C}/\text{min}$ with no hold, then to 240°C at a rate of $2^{\circ}\text{C}/\text{min}$ and held for 12 minutes. The column used a consistent flow rate of 1.2 mL/min by pressure ramping from 36 to 57 psi at 0.5 psi/min. A Supelco SP-2560 fused silica capillary column (100 mm length, 0.25 mm ID,

0.2 µm film coating thickness) was used for both analyses. The calibration standard was run every 5 samples to reconfirm the retention time. The standard mix for regular fatty acids consisted of 37-component FA standard (Supelco 47885-U) added with methyl stearidonate (Fluka 43959), 13-eicosenoic acid methyl ester (Sigma E3512), 9-eicosenoic acid methyl ester (Indofine Chemical 20-2001-1), 16-docosatetraenoic acid methyl ester (Sigma D3534) and 19-docosapentaenoic acid methyl ester (Supelco 47563-U). PMI fatty acids were quantified using the nearest-neighbour method as no standards are available, as in Mezek et al. (2011).