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Airborne polycyclic aromatic compounds contribute to the induction of the tumour-suppressing *P53* pathway in wild double-crested cormorants

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ABSTRACT

Polycyclic aromatic compounds (PACs), including polycyclic aromatic hydrocarbons (PAHs) and PAH-like compounds are known or probable environmental carcinogens released into the environment as a by-product of incomplete combustion of fossil fuels and other organic materials. Studies have shown that exposure to PACs in the environment can induce both genotoxicity and epigenetic toxicity, but few studies have related PAC exposure to molecular changes in free ranging wildlife. Previous work has suggested that double-crested cormorants (*Phalacrocorax auritus*; DCCO) exhibited a higher incidence of genetic mutations when their breeding sites were located in heavily industrialized areas (e.g., Hamilton Harbour, Hamilton, ON, Canada) as compared to sites located in more pristine environments, such as in Lake Erie. The aim of this study was to determine if airborne PACs from Hamilton Harbour alter the tumour-suppressing *P53* pathway and/or global DNA methylation in DCCOs. Airborne PACs were measured using passive air samplers in the Hamilton Harbour area and low-resolution mass spectrometry analysis detected PACs in livers of DCCOs living in Hamilton Harbour. Further hepatic and lung transcriptional analysis demonstrated that the expression of the genes involved in the DNA repair and cellular apoptosis pathway were up-regulated in both tissues of DCCOs exposed to PACs, while genes involved in p53 regulation were down-regulated. However, global methylation levels did not differ between reference- and PAC-exposed DCCOs. Altogether, data suggest that PACs activate the *P53* pathway in free-ranging DCCOs living nearby PAC-contaminated areas.

1. Introduction

Polycyclic aromatic compounds (PACs), including parent polycyclic aromatic hydrocarbons (PAHs), alkylated PAHs, naphthalene, and dibenzothiophenes (Achten and Andersson, 2015), are widespread environmental contaminants that are formed by the incomplete combustion of organic matter through natural (e.g., forest fires) and anthropogenic sources (e.g., burning of fossil fuels), or as components of petroleum products. Airborne PACs are generally associated with particulates in the air (including PM2.5; IARC, 2010) with deposition as the main route of entry of PAHs into the terrestrial and aquatic environment (Kim et al., 2013). Breathing in PAHs from ambient air, smoking, and diet are the main routes of PAH entry into the human body (ACGIH, 2005; reviewed in Kim et al., 2013). The International Agency for Research on Cancer classifies some PAHs as carcinogenic to humans and animals (Class 1), while other PAHs are classified as possibly carcinogenic (Class 2A, 2B; IARC, 2010).

Many studies have demonstrated that PACs and their metabolites are carcinogenic causing acute and chronic health effects in vertebrates. In human epidemiology studies, quantifiable links have been investigated between ambient exposure through occupation (e.g., coke oven workers) and the increased risk of lung cancer (reviewed in Boström et al., 2002). Mice exposed orally to benzo[*a*]pyrene had higher mRNA levels of cancer-related genes in lung tissue and most of these altered genes were involved in the tumour-suppressing *P53* pathway, including cell cycle arrest/senescence (*CDKN1A* or *P21*), apoptosis (*BAX*), and negative regulation of *P53* (*MDM2*; Labib et al., 2012). Moreover, *P53R2* (ribonucleotide reductase small subunit 2; also known as *RRM2B*) and *GADD45α* (growth arrest and DNA damage) have been validated as biomarkers of benzo[*a*]pyrene exposure to human cells (Ohno et al., 2008; Xin et al., 2015). In addition, reactive oxygen species formation due to PAH metabolite exposure can activate

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P53, which in turn, will augment *OGG1* (8-oxoguanine DNA glycosylase), a gene that repairs DNA damaged via base excision repairs (Abedin et al., 2013). Of note, in over half of human cancers, *P53* is mutated losing the ability to activate the tumour-suppressing pathways, which can lead to carcinogenesis (reviewed in Robles et al., 2002).

Furthermore, environmental contaminants can alter the stability of the genome through epigenetic modifications (Baccarelli and Bollati, 2009). DNA methylation occurs by DNA methyltransferases (DNMTs) adding a methyl group to cytosine followed by a guanine (CpG), creating 5-methylcytosine (5-mC; Pfeifer et al., 2014). Ten-eleven translocation (TET) proteins oxidize 5-mC to 5-hydroxymethylcytosine (5-hmC), an intermediate but stable state in the demethylation pathway (Pfeifer et al., 2014). A decrease in global 5-mC levels can lead to greater genomic instability and correlates with tumour progression through carcinogenesis (Kisseljova and Kisseljov, 2005). Alternatively, hypermethylation can occur, by increasing DNMT1 activity inducing 5-mC production, or by impairing the demethylation system through decreasing TET activity, leading to some cancers in humans (Pfeifer et al., 2014). Alteration of methylation of gene promotors has been used as a biomarker of lung carcinogenesis in humans (reviewed in Vineis and Husgafvel-Pursiainen, 2005). Other studies have clearly shown that environmental contaminants, including PACs, can alter DNA methylation levels in an experimental setting, including benzo[*a*]pyrene causing global hypomethylation in mice (Wilson and Jones, 1983) and zebrafish (Corrales et al., 2014; Fang et al., 2013). However, PACs exist in complex mixtures in the environment and can cause both hypomethylation and hypermethylation in humans (reviewed in Ruiz-Hernandez et al., 2015), but little is known on how environmental contaminants affect wildlife DNA methylation levels in natural populations (Head, 2014).

Air pollution, including airborne PACs has been shown to alter the health of wild birds. Cruz-Martinez et al. (2015) found that oil sands related air emissions resulted in induction of detoxifying enzymes and altered immune function in exposed tree swallows (*Tachycineta bicolor*). Concentrations of protoporphyrins, which are precursors to heme and to cytochrome P450 enzymes, from rock dove (*Columba livia*) excreta were proportional to airborne PAHs in the city of Milan, Italy (Sicolo et al., 2009). Although PAC emissions have been declining since the 1950s, the highest levels of PACs occur in industrial areas (Kim et al., 2013). Canada's two largest steel mills are located in Hamilton Harbour, Ontario and produce emissions containing complex mixtures of PACs. These compounds have been found at high concentrations in harbour sediment (1.6 – 1470 μg/g total PAH; CEPA, 1994) above the interim sediment quality guidelines ranging 0.006 – 0.1 μg/g dw for individual PAHs (Canadian Council of Ministers of the Environment, 1999). In addition, PACs have been detected at higher concentrations in harbour air (0.6–22.12 ng/m^3 sum of 8 PACs, average 1.22 ng/m^3 benzo[*a*]pyrene in 2013; HAMN, 2016) than the Ontario Ambient Air Quality Criteria guideline of 0.22 ng/m^3 benzo[*a*]pyrene (HAMN, 2016). The double-crested cormorant (*Phalacrocorax auritus*; DCCO) is an abundant piscivorous waterbird that breeds and lives in large colonies in Hamilton Harbour, ON. This bird population is continuously exposed to environmental PACs, including from the various steel mills and other large industry that dot the landscape and from vehicular exhaust from the two major highways adjacent to the harbour. A previous study done by King et al. (2014) detected PAHs in DCCO chick liver and bile from sites in Hamilton Harbour, including the mutagenic phase I metabolite benzo[*a*]pyrene-7,8-diol. In addition, microsatellite mutation rates were 11-fold higher in DCCOs sampled from a site (Pier 27) located downwind of the coking ovens of the steel mills than those from the reference site (King et al., 2014). Earlier work on herring gulls (*Larus argentatus*) indicated that germline DNA mutation rates were higher at Hamilton Harbour relative to cleaner reference groups, using minisatellites (Yauk and Quinn, 1996). Additionally, breathable particulate matter from Hamilton Harbour was found to be the casual factor behind the induction of germline mutations in caged mice (*Mus musculus*, Somers et al., 2004). These studies indicated that the increased rate of mutations may be a signal of DNA damage as a result of PAC exposure.

In addition to airborne exposure, diet is potentially another important route of PAC exposure. Indeed, DCCOs living in Hamilton Harbour are also being exposed from eating fish that are known to contain high levels of PACs coming from the Randle reef coal tar deposit; a site awaiting remediation and considered to be among the most polluted area in Canada (Hall and O'Connor, 2016). However, previous work has demonstrated that the diet of the different DCCO colonies living in Hamilton Harbour were virtually identical as estimated by regurgitates, fatty acid, and stable isotope profiles (King et al., 2014, 2017). The foraging range of DCCO is typically several km from their home colony (on average 2 km with a maximum of 40 km; Custer and Bunck, 1992) and they often forage outside of the harbour proper on a regular basis. This suggests that adult DCCO from Hamilton Harbour colonies, which are in close proximity to one another (< 3 km), likely forage for the same resources in the Lake Ontario area. Therefore, any differential biomarker effects in DCCOs from Hamilton Harbour colonies would likely be due to PAC differences in airborne exposure rather than diet.

The aim of this study was to determine if environmental exposure to complex mixtures of PACs alter the tumour-suppressing *P53* pathway and/or global DNA methylation rates in wild DCCOs. To do this, we determined mRNA, protein, and global methylation levels in liver and lung tissue of DCCOs living at sites with differing PAC airborne exposures. By estimating the expression of genes associated with *P53*, we can assess if PAC airborne exposure alters the tumour-suppressing *P53* pathway, which potentially could lead to carcinogenesis. We predicted that DCCO living in Hamilton Harbour and downwind to the source of PACs would have the most alterations to the *P53* pathway and DNA methylation levels than DCCO living on the reference site in Lake Erie.

2. Methods

2.1. Sample collection

Ten double-crested cormorant (DCCO) chicks were sampled at each location: Mohawk Island in eastern Lake Erie (reference site; 42.8345° N, 79.5226° W), Centre Island (43.3046° N, 79.8028° W) and Pier 27 (43.2833° N, 79.7937° W) in Hamilton Harbour. DCCO colonies were visited in mid-June 2014. All sampling was restricted to prefledgling DCCO chicks, thus ensuring that all PAC exposure was from local sources either through the air or through diet via the parents. DCCO chicks were removed from the nest by hand and placed in a dark, covered box until processed. Chicks were decapitated following standardized operating protocols and animal care standards (Environment and Climate Change Canada ACC SOP-07). The bodies were immediately dissected to obtain lung and liver tissues that were targeted for assessing the *P53* pathway. The liver is generally considered the primary organ responsible for metabolism of xenobiotics because it contains the largest concentration of cytochrome P450 compared to other tissues. Lung tissue was collected, as it is the first point of contact with airborne exposures. All samples were flash frozen in liquid nitrogen and stored at −80 °C until genetic analysis.

2.2. Characterization of exposure

Exposure of DCCO to PACs were estimated in three ways: i) airborne exposure was assessed by measuring PACs using passive air samplers near the colonies in Hamilton Harbour, ii) air speed and velocity were measured up- and down-wind of the major putative PAC sources in Hamilton Harbour, relative to the location of the DCCO colonies, and iii) hepatic burdens of PACs were measured. Pier 27 was considered, putatively, downwind of steel mills and other heavy industry more often than Centre Island. Pictures of the two Hamilton Harbour colonies, with the terrain immediately upwind (WSW) of each can be seen