Epigenetic responses to oil exposure in wild Arctic seabird populations

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Abstract

Anthropogenic pollution has been shown to have detrimental effects on organismal physiology, behavior, and fitness, but the underlying genomic mechanisms mediating these effects are not well understood. Epigenetic regulation, such as DNA methylation, has been proposed as a potential mechanism mediating these effects but currently there are few studies in natural populations. Here, I examined the methylation patterns of liver tissues from black guillemot (*Cepphus grylle*) in regions in the Canadian Arctic with different histories of exposure to polycyclic aromatic compounds (PACs). When compared to a reference site, I observed that all three sites with PACs exposure share a core set of differentially methylated regions (DMRs), implying that there are some consistent methylation responses to these compounds. However, the two sites with shortterm exposure to anthropogenic sources of PACs shared more DMRs than they did with the site experiencing chronic exposure to natural PACs. Furthermore, I found that when compared to a reference site with very low PACs exposure, populations that have been exposed to anthropogenic PACs are characterized by having DMRs with significantly greater ratios of hypermethylated to hypomethylated versus the population experiencing chronic exposure to natural PACs. Taken together, these results imply that the specific composition and exposure length of PACs might influence the direction of the epigenetic response. This study provides novel insights into the epigenetic mechanisms underlying biological responses to anthropogenic oil pollution. The identified DMRs serve as a genomic resource for further research investigating the functional role of DNA methylation in response to anthropogenic oil pollution.

Résumé

La pollution d'origine anthropique a été démontrée comme ayant des effets néfastes sur la physiologie, le comportement et la fitness des organismes, mais les mécanismes génomique sousjacents à ces effets ne sont pas bien compris. La régulation épigénétique, telle que la méthylation de l'ADN, a été proposée comme un mécanisme potentiel médiant ces effets, mais actuellement, il existe peu d'études dans les populations naturelles. Dans cette étude, j'ai examiné les schémas de méthylation des tissus hépatiques du guillemot à miroir (Cepphus grylle) dans des régions de l'Arctique canadien présentant des histoires différentes d'exposition aux composés aromatiques polycycliques (CAP). Par rapport à un site témoin, j'ai observé que les trois sites exposés aux CAP partagent un ensemble central de régions différentiellement méthylées (DMRs), ce qui implique qu'il existe des réponses de méthylation cohérentes à ces composés. Cependant, les deux sites exposés à une exposition à court terme à des sources anthropiques de CAP partagent plus de DMRs entre eux qu'avec le site soumis à une exposition chronique aux CAP naturels. De plus, j'ai constaté que, par rapport à un site de contrôle sans exposition aux CAP, les populations exposées à des CAP d'origine anthropique se caractérisent par des DMRs avec des ratios significativement plus élevés de hyperméthylation par rapport à l'hypométhylation par rapport à la population exposée chroniquement aux CAP naturels. Dans l'ensemble, ces résultats impliquent que la composition spécifique et la durée d'exposition aux CAP peuvent influencer la direction de la réponse épigénétique. Cette étude offre de nouvelles perspectives sur les mécanismes épigénétiques sousjacents aux réponses biologiques à la pollution pétrolière d'origine anthropique. Les DMRs identifiées servent de ressource génomique pour des recherches ultérieures visant à étudier le rôle fonctionnel de la méthylation de l'ADN en réponse à la pollution pétrolière d'origine anthropique.

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Author contributions

Wing-Zheng Ho and Rowan Barrett conceived the study. Reyd Smith and Frederic Dwyer-Samuel collected the samples. Wing-Zheng Ho and Åsa Lind performed lab work. Wing-Zheng Ho analyzed the data. Wing-Zheng Ho and Rowan Barrett wrote the manuscript.

List of Abbreviations

PACs: Polycyclic aromatic compounds

PCB: Polychlorinated biphenyl

DMRs: Differentially methylated regions

USEPA: U.S. Environmental Protection Agency

IARC: Agency for Research on Cancer

CCME: Canadian Council of Ministers of the Environment

HMW: High molecular weight

LMW: Low molecular weight

WGBS: Whole genome bisulfite sequencing

ECCC: Environment and Climate Change Canada

PCA: Principal Component Analysis

PCs: Principal components

Lbfabp: Liver basic fatty acid binding protein 1

Lipc: Hepatic lipase

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Introduction

Marine biodiversity makes a significant contribution to the ecosystem functions necessary for life on Earth (Thurber et al., 2014), including food production (Costello et al., 2020), nutrient cycling, and carbon absorption (Folkersen et al., 2018). Rising temperatures and ocean acidification driven by climate change are disrupting marine ecosystems by driving range shifts (van Putten et al., 2016), driving keystone species extinct (Hamilton et al., 2015) and increasing mortality rates (Rühmkorff et al., 2023). The marine habitats and species in Arctic regions are especially susceptible to environmental changes (Michel et al., 2012). With decreases in the extent of Arctic sea ice in Northern Canada, there have been increases in anthropogenic activity in this region, notably in the form of ship traffic, which has led to increases in the frequency of oil spill events (Mudryk et al., 2021). Oil contaminants such as polycyclic aromatic compounds (PACs), a group of organic pollutants produced from incomplete combustion of organic materials, are a potent stressor that can cause widespread damage to local organisms, ranging from lethal effects via acute exposure to long-term effects via sublethal chronic exposure, leading to health, growth or reproductive problems in affected individuals (e.g., in tuna, common murres, south polar skuas, and Magellanic penguins; Brette et al., 2014; Fry et al., 1985; Eppley Z. A. and Rubega M. A., 1990; Fowler et al., 1995). PACs exposure can lead to a variety of physiological and reproductive issues. Exposure to PACs has been extensively studied in fish, leading to the identification of various adverse effects on both freshwater and marine species, revealing cardiovascular issues like cardiac arrhythmias and circulatory failure (Incardona, 2017), as well as cardiotoxicity (Ainerua et al., 2020; Clark et al., 2010; Zhang et al., 2013), and endocrine disruption (Lahnsteiner et al., 2005) in fish. While the mechanisms of PACs toxicity are less well-studied in other taxa, similar embryotoxic responses as observed in fish have been detected. For instance, the PACs component of crude oil has been shown to be toxic to the embryos of Mallard ducks (*Anas platyrhynchos*). The exposure resulted in various malformations, hindered growth, and an increased death rate after the formation of the chorioallantoic membrane within the egg (Hoffman and Gay, 1981). Additionally, a study on African clawed frog (*Xenopus laevis*) demonstrated that PAC-exposed embryos had reduced length and slower development compared to controls (Bryer et al., 2006). These effects can have severe population consequences, as have been documented by the delayed recovery of harlequin duck (Iverson, S. A., and Esler, D., 2010) and sea otter (Esler et al., 2018) populations for 14 and 20 years after oil spills, respectively. However, while the short-term physiological impacts of oil spills have been well studied, the underlying genomic mechanisms mediating these effects are not as well understood (but see Crump et al., 2016; Head et al., 2015; Staal et al., 2008; Zahaby et al., 2021).

Among the hundreds of PACs, 16 compounds have been identified as priority contaminants by organizations such as the U.S. Environmental Protection Agency (USEPA), the International Agency for Research on Cancer (IARC), and the Canadian Council of Ministers of the Environment (CCME). These PACs have two or more aromatic rings, are non-polar, stable, and hydrophobic, making them highly resistant to biodegradation and more likely to accumulate in soil or water (Adeola and Forbes, 2021; Alaba et al., 2018; Lemaire et al., 2019; Sullivan et al., 2019). High molecular weight (HMW) PACs with four or more aromatic rings are more carcinogenic or mutagenic than the low molecular weight (LMW) PACs with fewer aromatic rings (Figure 1; Costa et al., 2017; Shi et al., 2018; USEPA, 2000). However, in contrast to HMW PACs, LMW PACs tend to remain in solution and are readily available to marine organisms through ingestion or respiration, making them more toxic for marine biota, and are mostly associated with acute toxicity

and genotoxicity rather than carcinogenic properties (National Toxicology Program, 2011). The solubility of LMW PACs also increases with temperature, making them more bioavailable in warmer seasons (Neff, 1979; National Research Council Canada Environmental, 1983). Additionally, LMW PACs have a lower number of aromatic rings, and because the potential for bioaccumulation in marine biota increases with octanol-water partition coefficients, which are proportional to the number of aromatic rings (Gobas et al., 2003), LMW PACs have lower potential of bioaccumulation despite their higher bioavailability relative to HMW PACs. In the context of pyrogenic and petrogenic PACs, the origin of these compounds significantly influences their environmental impact and toxicity. Pyrogenic PACs, produced from incomplete combustion of organic materials or processed oil, often carry a higher proportion HMW PACs, influencing their toxicity profile and environmental fate (Stout et al., 2001). These distinctions between LMW and HMW PACs highlight the importance of considering the specific characteristics of different PACs when assessing their potential impact on the environment.



Figure 1. Structures of the 16 USEPA priority pollutants PACs. (Source: Chemistry Matters)

The specific response of organisms to environmental stressors can also be influenced by their timescale of exposure. Long-term, gradual exposure can often provide greater opportunities for adaptation, and reduce the likelihood of extinction (Bell and Gonzalez 2009; Collins and De Meaux, 2009). Whereas slow rates of environmental change may allow for selection of beneficial mutations and changes in gene expression that contribute to adaptive responses in local populations (Bell, 2013, 2017; Flores et al., 2013; Harmon and Pfennig, 2021; Morgan et al., 2007; Samani and Bell, 2016; Vanselow et al., 2022), more rapid rates of environmental change can make acclimation or adaptation more difficult, and lead to potentially detrimental phenotypic endpoints (Bay et al. 2017; Dolinoy et al., 2007; Turner, 2009). As anthropogenic activities expose natural populations to increasingly variable and extreme changes in environmental conditions (Eyer et al. 2019, Hu et al. 2018, IPCC 2018, Stott 2016, Walther et al. 2002), it is crucial to understand the

distinct mechanisms that might permit populations to respond to diverse perturbation scenarios, ranging from chronic to acute exposure.

Epigenetic mechanisms, including histone modification, ncRNA, and DNA methylation, can play a key role in rapid responses to environmental stressors (Dutta et al., 2018; Kilvitis et al., 2017; Lim et al., 2021; Verhoeven et al., 2016) and could potentially serve as a useful indicator of the impacts of oil contamination in marine wildlife. In particular, DNA methylation, which is the addition of a methyl group onto a cytosine and is usually associated with downregulation of gene expression, has been well studied and shown to be a reliable epigenetic marker for environmental stressors (Cao Yi, 2015; Jeremias et al., 2022; Moore et al., 2013; Skinner et al., 2018; Valdivieso et al., 2023; Virani et al., 2016). Beyond serving as an indicator (Gouin et al., 2023; Koch and Wagner, 2011; Moore et al., 2013; Ye et al., 2022), DNA methylation has a profound impact on gene expression by impeding the binding of transcriptional activators to DNA and altering chromatin states to inhibit transcription factor binding (Moore et al., 2013; Rose & Klose, 2014). This process includes hypermethylation, leading to transcriptional silencing due to methylation of the 5'-carbon of the cytosine aromatic ring, which typically represses gene expression, especially when methylated sites are proximal to the transcription start site in the promoter region (Bird, 2002; Laine et al., 2016; Li et al., 2011; Moore et al., 2013). However, it is challenging to generalize the relationship between DNA methylation and gene expression since DNA methylation can also enhance transcription (Korochkin, 2006). DNA methylation and its converse process, demethylation, play a role in regulating phenotypic traits, thereby mediating phenotypic trait expression (Law and Jacobsen, 2010). With advances in DNA methylation sequencing methods such as whole genome bisulfite sequencing (WGBS), it is becoming feasible to investigate how

anthropogenic pollutants can induce changes in DNA methylation in wild populations of animals (Chen et al., 2021, Hu et al., 2021, Laine et al., 2021, Zhang et al., 2021). WGBS is a comprehensive method for studying DNA methylation patterns at single-base resolution across the entire genome (Harris et al., 2010). WGBS involves treating DNA with bisulfite to convert unmethylated cytosines to uracil, while leaving methylated cytosines unchanged (Figure 2). Unmethylated cytosines will appear as thymines, while only methylated cytosines will appear as cytosines in the resulting sequences. Variation in DNA methylation may provide a mechanism to avoid declines in fitness when individuals are exposed to environmental changes (Janowitz Koch et al., 2016). These changes can also act on shorter timescales than genomic adaptation (Bossdorf et al., 2008) and may persist across generations (Head, 2014). However, the relevance of epigenetic responses to varying timescales of oil exposure in natural populations remains unclear. Additionally, I am not aware of many studies that have directly compared the functional consequences of exposure to distinct PACs types on the epigenome in natural populations outside of the laboratory.



Figure 2. General principle of WGBS consists of bisulfite modification of genomic DNA, followed by the creation of a sequencing library. Treatment of DNA with bisulfite converts cytosine residues to uracil, but leaves 5-methylcytosine residues unaffected. Variants may switch the order of bisulfite conversion and library preparation. (Masser et al., 2018)

Research investigating DNA methylation changes induced by oil exposure in wild animals has generally focused on global methylation responses rather than site-specific methylation changes, with these global shifts showing inconsistent patterns across studies. For example, a study in juvenile red drum found significant associations between high PACs exposure and hypomethylation in global methylation levels (Cañizares-Martínez et al., 2022), whereas a study on double-crested cormorants found no significant association between airborne PACs exposure and global methylation levels (Wallace et al. 2018). However, given the different PAC exposure routes, PAC concentrations, and study species, perhaps it is unsurprising to find inconsistency in these global methylation patterns. Analyzing site-specific methylation level differences can be useful because it enables the identification of differentially methylated regions (DMRs) that are associated with variation in ecologically relevant phenotypes and behaviors (Schrey et al., 2012). Site-specific methylation data can help to identify differentially methylated CpG sites within specific gene regions, such as promoter regions or enhancers, which may be particularly important for regulating gene expression (Hu et al., 2014; Ko et al., 2013). Therefore, techniques such as WGBS can be valuable by permitting detection of CpG loci and the analysis of DNA methylation at single-base resolution across the genome. (Beck et al., 2022).

Here, I used WGBS to investigate whether exposure to natural and anthropogenic oil pollutants alters DNA methylation in populations of a wild seabird, the black guillemot (*Cepphus grylle*; Figure 3). The black guillemot is a valuable model species in ecological and environmental studies, given its broad distribution across Northern Atlantic and Arctic regions, and its sensitivity to environmental changes (Piatt et al., 2007). The black guillemots native to the eastern region of Baffin Island are hypothesized to travel distances ranging from several tens to hundreds of

kilometers away from their colony during winter. However, the exact migratory patterns of this species remain largely unknown (Butler et al., 2020). During breeding season, black guillemots gather in dense colonies, making them highly amenable for monitoring and sample collection for contaminants research, and allowing them to serve as useful indicators of oil exposure at the ecosystem level (Piatt et al., 2007). The species' dietary reliance on benthic prey makes it vulnerable to marine pollutants as they dive to a depth range of 15-18m and forage up to 15km (Shoji et al., 2015), with previous studies observing guillemot population declines correlating with increased polychlorinated biphenyl (PCB) levels (Hoffman et al., 1996; Kuzyk et al., 2003). External oil impairs bird feather functionality, causing hindered flight and potentially increasing predation risk, while also compelling the birds to spend more time on cleaning activities, thereby disturbing their normal routines (Maggini et al., 2017; Henkel et al., 2014). Ingested oil toxins can lead to physiological distress and delays in migration due to increased energy expenditure (Bianchini and Morrissey, 2018; Maggini et al., 2017). The negative impacts of oil contaminants also extend to reproduction, with observations of decreased parental care and reduced chick weight gain (Miller et al., 1978). Furthermore, PACs can disrupt hormone regulation, leading to depressed growth in oil-dosed birds (Peakall et al., 1981). Exposure to PACs has also been linked with genotoxic effects, causing DNA damage (Laffon et al., 2006). At a cellular level, exposure to PACs can influence cellular metabolism and induce oxidative stress, leading to cytotoxicity (Leighton et al., 1983). Previous studies also demonstrate the utility of the black guillemot in studies of climate change impact, with changes in sea ice conditions found to influence the species' feeding habits and reproductive success (Divoky et al., 2021; Varpe and Gabrielsen, 2022). Thus, the black guillemot offers a versatile model for examining anthropogenic impacts on marine ecosystems and providing insights into pollutant effects and climate-related changes.



Figure 3. (A) The black guillemot (*Cepphus grylle*). (B) A black guillemot diving. (Source: "OCEAN TREASURES" Memorial Library)

In June 2020, during a period when black guillemots, key predators in these marine ecosystems, were either actively in nesting phase or preparing to nest, a spill of approximately 3000L of crude oil occurred in Postville, Nunatsiavut (Environment and Climate Change Canada [ECCC], personal communication, October 2021). The event had significant implications for black guillemots in the area, with the total mean concentration of the 16 USEPA priority PACs in livers of birds reaching 40.71 ng/g lipid weight (see Methods). The high mobility and foraging methods of these birds, which often involves diving into the water to catch their prey, means that they are likely to encounter oil if it is present on the water's surface or sub-surface (Henkel et al., 2012; Wiese and Ryan, 2003). In addition to the spill site at Postville, I leveraged three additional sites in Arctic Canada (one other in Nunatsiavut and two in Nunavut) for comparisons in this study (Table 1; Figure 4). I selected Nain because it represents a site where PACs exposure is elevated in relative to the reference site due to higher levels of vessel traffic (Figures 5-7; Arctic Monitoring and Assessment Programme, 2010; Harsem et al., 2011; Pizzolato et al., 2013). The site is thus characterized by a higher ratio of low-molecular weight PACs to high molecular weight PACs (Jennifer Provencher, unpublished data), but without the presence of an acute stress event such as

an oil spill. Nain therefore represents a chronic but lower level of exposure to anthropogenic PACs relative to Postville, while sharing a similar PACs composition. In contrast, Qikiqtarjuaq represents a site exposed to natural hydrocarbon seeps, and is characterized by a higher ratio of high-molecular weight PACs (Provencher et al., 2020). Black guillemots at this site therefore experience chronic exposure to PACs with a different composition than those present at Postville and Nain (a higher ratio of high-molecular weight PACs). Finally, Pond Inlet was selected as a reference site due to a lack of natural oil seeps (Geological Survey of Canada, 2022; van Luijk et al., 2020) and low vessel traffic as extensive sea ice coverage in the summer limits the accessibility and navigability of vessels (Haas and Howell, 2015; Howell and Brady, 2019) at Pond Inlet and Qikiqtarjuaq, which is not the case for Postville and Nain (Figures 5-7).

My objectives are twofold. First, I aimed to identify how the source of oil pollution (anthropogenic for Nain and Postville, and natural for Qikiqtarjuaq) impacted the methylation response of exposed seabirds. Second, I aim to compare how the methylation response of seabirds differs when exposed to a sudden, acute oil pollution event (the spill at Postville) versus a chronic but lower level of oil exposure due to anthropogenic activity (vessel activity at Nain). By identifying DMRs that are unique to these different scenarios, my research will contribute novel understanding about the epigenetic response mechanisms used by wild populations of animals exposed to anthropogenic stressors.

Site	Site type	Vessel based oil pollution	Natural seep oil
Pond Inlet,	REFERENCE	Low vessel traffic (Figure 5; Pizzolato et	No known oil seeps near the breeding
Nunavut		al., 2014), no known acute commercial	colonies (Bennett et al., 2014; Foster et
		vessel oil spills in the region (NIRB,	al., 2015; NIRB, 2018).
		2018).	
Qikiqtarjuaq,	SEEP	Low vessel traffic (Figure 5; Pizzolato et	Natural oil and gas seeps have been
Nunavut		al., 2014), no known acute commercial	reported by Inuit harvesters and
		vessel oil spills in the region (NIRB,	research vessels (Bennett et al.,
		2018).	2014; Foster et al., 2015; NIRB, 2018).
Nain, Nunatsiavut	SHIP	Moderate vessel traffic in the region	No known oil seeps near the breeding
		(AMSA 2009; Figure 6), no known acute	colonies (Jauer and Budkewitsch,
		commercial vessel oil spills in the region.	2010).
Postville,	SPILL	Moderate vessel traffic in the region	No known oil seeps near the breeding
Nunatsiavut		(AMSA 2009; Figure 6), documented	colonies (Jauer and Budkewitsch,
		acute spill of 3000L of oil in the region in	2010).
		June 2020 (Environment and Climate	
		Change Canada [ECCC], personal	
		communication).	

 Table 1. Characteristics of each study site.



Figure 4. Locations black guillemot colonies where liver samples were collected.



Ship traffic from 2012 to 2019 and minimum sea-ice extent from 1990 to 2019 in the Polar Regions

Figure 5. Observed vessel traffic along the Arctic maritime trade routes (Northwest Passage, Transpolar Route and Northern Sea Route) from 2012 to 2019. (Constable et al., 2022)



Figure 6. Snapshots of Vessel Traffic Based Automatic Identification System (AIS) Data showing the difference in ship vessel traffic in Pond Inlet and Nain due to difference in sea ice coverage in (A) June and (B) September. Source: Global Maritime Traffic

Materials and Methods

Liver sampling protocol, DNA extraction and WGBS library preparation

In collaboration with Environment and Climate Change Canada, I obtained fresh liver samples from bird carcasses of black guillemot (Cepphus grylle), which were collected, and flash frozen on-site. As described in detail in Provencher et al. (2020), teams of local Inuit hunters and researchers collected the seabirds using 12-gauge shotguns or 22 caliber rifles while the birds were away from the breeding colonies and feeding on the water. In October 2022, birds were collected from a reference site with very low PACs exposure (Pond Inlet: REFERENCE), a site of a recent oil spill (Postville: SPILL) in October 2020, and a site with higher ship traffic (Nain: SHIP) in October 2020. Birds were also collected from a site with natural oil and gas seeps (Qikiqtarjuaq: SEEP) in August 2018. The collected birds are of unknown sex, unbanded, and were not incubating eggs at the time of collection (Jennifer Provencher, personal communication). Birds from each site show evidence of distinct exposure histories to PACs, as reflected by clear differences in the mean concentration of LMW and HMW PACs in their livers. The total mean concentration of the 16 USEPA priority PACs for REFERENCE (1.89 ng/g, SD = 4.21) is 21.54 times lower than SPILL (40.71 ng/g, SD = 106.68), 6.23 times lower than SHIP (11.77 ng/g, SD = 12.19), and 49.56 timeslower than SEEP (93.67 ng/g, SD = 408.93) (Figure 7). The ratio of LMW to HMW PACs is 1.44in REFERENCE, 1.34 in SPILL, 2.11 in SHIP, and 0.63 in SEEP (Jennifer Provencher, unpublished data; Provencher et al., 2020), showing that LMW PACs constituted the majority of the total PACs burden for birds at both SPILL and SHIP sites, while HMW PACs were in the majority at the SEEP site. Considering that various PACs are rapidly metabolized by organisms (Shilla and Routh, 2018), the detected concentrations most likely represent exposure to these

compounds within a few days prior the collection of the birds in their foraging zones around the breeding colonies. Samples were stored in a -80C freezer prior to extraction. DNA and RNA of samples of black guillemot (n = 16 from Nain, 14 from Postville, 28 from Qikiqtarjuaq, 21 from Pond Inlet) were extracted using QIAGEN AllPrep DNA/RNA mini kit (Qiagen, Hilden, Germany) following manufacturer's instructions. The DNA samples were submitted to the McGill Genome Center for whole genome bisulfite sequencing (WGBS) library preparation and sequencing on the Illumina NovaSeq 6000 Sequencing System at 15× target coverage for each lane per population.



Figure 7. Box plot showing the sum of Log_{10} transformed mean total PACs concentration in liver tissue of black guillemot in site REFERENCE, SPILL, SHIP and SEEP.

WGBS data processing and identification of differentially methylated sites and regions

I pre-processed the raw sequencing data using the bioinformatics analysis pipeline methylseq v.2.3.0 (Ewels et al., 2022) through the workflow framework nf-core (Ewels et al., 2020). I first performed quality check on the raw sequencing reads using FastQC v.0.11.9 (Andrew, 2010), then trimmed adapter sequences and low-quality sequences using Trim Galore! v.0.6.7 (Krueger et al., 2021). I then mapped the processed reads to the black guillemot genome assembly (ASM1340106v1; NCBI BioProject PRJNA545868), deduplicated, and extracted the methylation call data using Bismark v.0.24.0 (Krueger and Andrews, 2011). On average, each sample yielded 1×10^8 raw reads, and after quality filtering, I retained 1×10^8 reads. On average, I found that 7×10^7 (73.1%) of the quality-filtered reads uniquely mapped against the black guillemot genome assembly. In total, I analyzed an average of 3×10^9 cytosine bases.

I assessed the DNA methylation differences in each seabird population from the three exposed sites (SPILL, SHIP, and SEEP) relative to the REFERENCE site. I identified the CpG loci using the R package methylKit v.1.24.0 (Akalin et al., 2012) by importing the extracted methylation call data from Bismark via the function *methRead*. I then filtered the CpG loci for a minimum coverage threshold of 5 (lo.count = 5) and a maximum of 100 reads per base (hi.count = 100), and excluded bases in the 99.9th percentile of coverage (high.perc = 99.9) using the *filterByCoverage* function, and normalized the filtered reads using the *normalizeCoverage* function to prevent any potential PCR bias. I used the function unite with the option destrand set to true to merge all CpG loci such that they are covered in at least 80% of samples per group. I then used these CpG loci to calculate

differential methylation via the function *calculateDiffMeth*, which is a logistic regression model that models the log odds ratio based on methylation proportion of a CpG. I then identified the differentially methylated regions (DMRs) using the function *callDMR* in the R package DSS v.2.47.1 (Feng and Wu, 2019) with the default parameters: minimum length of 50bp for DMRs, minimum 3 CpG sites for DMRs and minimum percentage of CpG sites with significant p-values (≤ 0.01) in DMRs at 50%, consistent with previous studies (Jeremias et al., 2018; Skjærven et al., 2018; Wang et al., 2021). I assessed the statistical significance of differences in the number of DMRs between study sites using a Pearson's Chi-Squared test via the function prop.test in R v.4.1.3 (R Core Team, 2021), and differences in the ratios of methylation status (hyper- versus hypomethylated) using a Fisher's Exact Test. Furthermore, I also checked for any overlapping DMRs between each comparison pair, and between all three comparisons, using the genomic intersection tool Intervene v.0.6.5 (Khan and Mathelier, 2017) and the *intervene venn* command. Statistical significance of the overlap between pairs of study sites, all three sites, and the differences between the number of overlaps between comparisons were each calculated with a 10,000 round permutation test. All statistical tests are based on a heuristic that uses the mean length of regions in our sets of DMRs (~400 bp) and the size of the genome (~ 1.3×10^9 bp) to calculate the total number of possible DMRs ($\sim 3 \times 10^6$ bp).

Results

General DNA methylation patterns

In each site, approximately 4.5% of all genomic C sites were methylated. The overall genomewide levels of CG, CHG and CHH methylation is 63.3%, 0.6% and 0.6%, respectively for REFERENCE, 67.5%, 0.7% and 0.7%, respectively for SPILL, 65.6%, 0.7% and 0.7%, respectively for SHIP, 66.9.3%, 0.6% and 0.6%, respectively for SEEP (Figure S1). The proportions of these methylation types were similar in each site, 87% CG, 3% CHG, and 10% CHH in REFERENCE, 86% CG, 4% CHG, and 10% CHH in SPILL, 87% CG, 3% CHG, and 10% CHH in SHIP, and 88% CG, 3% CHG, and 9% CHH in SEEP (Figure S2).

Different mechanisms of response between sites with different exposure histories

I found that the oil exposed populations varied in their number of differentially methylated regions relative to the reference population, with the sites exposed to the oil spill and chronic natural hydrocarbon seepage showing a greater number of DMRs than the site exposed to PACs via chronic shipping traffic (Pearson's Chi-Squared test: REFERENCE-SPILL vs. REFERENCE-SHIP, $\chi 2 = 40.675$, df = 1, p $\leq 1 \times 10^{-10}$; REFERENCE-SEEP vs. REFERENCE-SHIP, $\chi 2 = 52.99$, df = 1, p $\leq 1 \times 10^{-10}$). I identified 382 DMRs in the REFERENCE-SPILL comparison, 224 DMRs in the REFERENCE-SHIP comparison, and 408 DMRs in the REFERENCE-SEEP comparison (Figure 8). The direction of differential methylation varied according to the collection site of the

birds, with higher ratios of hypermethylation in sites exposed to anthropogenic PACs compared to the site SEEP exposed to natural PACs (Fisher's Exact Test, $p \le 1 \times 10^{-10}$). In the REFERENCE-SPILL comparison I identified 95.03% of the 382 DMRs as being hypermethylated and 4.97% being hypomethylated. Similarly, in the REFERENCE-SHIP comparison I identified 93.75% of the 224 DMRs as being hypermethylated versus 6.25% hypomethylated. In contrast, in the REFERENCE-SEEP comparison I found that 38.48% of the 408 DMRs were hypermethylated whereas 61.52% were hypomethylated (Figure 8).

Shared and distinct patterns of methylation across sites

I found significantly more shared DMRs between sites than would be expected by chance, with a total of 51 (8.42%; Permutation test, $p \le 1 \times 10^{-10}$), 33 (4.18%; Permutation test, $p \le 1 \times 10^{-10}$) and 33 (5.22%; Permutation test, $p \le 1 \times 10^{-10}$) shared between REFERENCE-SPILL and REFERENCE-SHIP, between REFERENCE-SPILL and REFERENCE-SHIP and REFERENCE-SEEP, respectively (Figure 9). In total, 16 DMRs were shared between all three comparisons with the reference site (Permutation test, $p \le 1 \times 10^{-10}$). However, the proportion of shared DMRs across sites was also associated with the type of PACs exposure, with more DMRs shared between the two sites exposed to anthropogenic PACs versus the comparison of each of these sites to the site exposed to natural PACs (Permutation test, $p \le 1 \times 10^{-10}$). I used Principal Component Analysis (PCA) to explore the variation in methylation patterns between the different sites. The first two principal components (PCs) explained 15.94% of the variation in the REFERENCE-SPILL comparison, 14.76% in the REFERENCE-SHIP comparison, and 13.91% in the REFERENCE-SEEP comparison when analyzing all CpG loci

with 5x coverage across samples in the PCA (Figure S3A, S4A, S5A). When analyzing only DMRs in the PCA, the first two PCs explained 49.77%, 55.97%, and 53.03% of the variation in the REFERENCE-SPILL, REFERENCE-SHIP, and REFERENCE-SEEP comparisons, respectively (Figures S3B, S4B, S5B). In the PCA using only DMRs, there is clear separation between the exposed sites and the reference site in all three comparisons, whereas no clear separation was observed when analyzing all CpG loci. In a PCA analyzing all four sites together, the first two principal components (PCs) explained 10.07% of the variation for all CpG loci (Figure S6A), and 16.97% of the variation when using only DMRs. In the PCA analyzing DMRs only, there is some separation between the REFERENCE and SEEP sites versus the SHIP site and SPILL site (mainly on PC1) (Figure S6B). Similarly, in a hierarchical clustering run on all CpG loci, SPILL and SHIP sites largely group together, as do REFERENCE and SEEP sites (Figure S7).



Figure 8. Distribution of hypo- & hypermethylated DMRs between each PAC-exposed site and reference.



Figure 9. Venn diagram of DMRs shared between SPILL, SHIP and SEEP sites vs. REFERENCE.

Discussion

Arctic biodiversity is under threat due to the increasing risk of anthropogenic PACs pollution, causing both acute and chronic damage to the ecosystem (Frederiksen et al., 2019; Helle et al., 2020). Epigenetic patterns may provide insights into the underlying genomic mechanisms mediating physiological responses to PAC pollution, as well as potentially serving as a useful indicator of the exposure history experienced by natural populations. I used the black guillemot as a study system to test for differences in patterns of methylation between three sites exposed to varying types and exposure length of PACs to a reference site, including an oil spill site, a high shipping traffic site, and a site with natural seepage of PACs. In this methylome-wide analysis, I found that approximately 4.5% of the cytosine sites were methylated in the black guillemot genome. Like data published in other species such as humans (Lister et al., 2014), sheep (Zhang et al., 2017), pigs (Hao et al., 2016), mice (Wei et al., 2020) and other birds (Han et al., 2020; Shi et al., 2021; Xu et al., 2022), CG methylation accounted for the highest proportion of methylation types. Additionally, I discovered that the liver tissues of individuals exposed to different levels and types of PACs contaminants possess methylome signatures with some shared characteristics but also distinctive aspects. Specifically, I identified a core set of DMRs that overlapped between all sites exposed to higher concentrations of PACs than at the reference site, suggesting some consistent epigenetic mechanisms of response in black guillemots. Moreover, the number of DMRs shared between the two sites exposed to anthropogenic sources of PACs (shipping traffic and oil spill) are significantly higher than the number of DMRs shared between these sites and the site exposed to natural PACs. This observation is further corroborated by the results of the hierarchical clustering, which shows grouping of the two anthropogenic PAC-exposed sites, separate from the cluster formed by the reference site and the site exposed to natural PACs. This

suggests there are likely to be certain methylation responses that are specific to exposure to anthropogenic, LMW PACs.

Further supporting the idea that black guillemots might be utilizing distinct epigenetic responses to natural versus anthropogenic sources of PACs are the significant differences in ratios of hyper versus hypomethylation between exposed sites versus the reference. I found that DMRs at the SEEP site primarily reflected hypomethylation relative to the reference, whereas DMRs at the SPILL and SHIP sites overwhelmingly showed hypermethylation. The results from the SEEP site are consistent with previous studies that have reported hypomethylation patterns associated with exposure to HMW PACs (Quintanilla-Mena et al., 2020; Shugart, 1990; Teneng et al., 2011). It has been suggested that hypomethylation of gene promoters might allow increased expression of genes involved with stress response (Cavalli and Heard, 2019; Metzger and Schulte, 2016). In contrast, black guillemots at Postville and Nain appears to have led to widespread hypermethylation. However, it is important to note that these findings should be interpreted with caution. As the study was conducted on wild populations, variability in DMRs between sites could also be influenced by a host of factors including, but not limited to, breeding status, migratory behaviors, diet, and individual metabolism of PACs. The collection of the birds is conducted in the late August to October period, after the breeding season so the birds were not incubating eggs, given that the collection date and breeding status of the birds are consistent between sites, these factors were most likely not in play. Furthermore, while the data on diet difference between site was not available, all the birds were in good condition, so direct starvation would not be a factor (Jennifer Provencher, personal communication). Genetic variability between populations at different sites and exposure to other environmental stressors could also potentially impact the

number of DMRs. I am not aware of any differences in any of these factors between sites, but it is possible that the observed methylation patterns could be the result of a stress response to these factors, rather than directly caused by PAC exposure history. In this study, the classification of PAC composition for each exposed site is simplified to LMW PACs for the anthropogenic sources and HMW PACs for the natural source based on population level data. Future studies could consider a correlation analysis with individual PACs exposure data and methylation profiles. This analysis together with detailed knowledge of genetic and environmental variability between the populations and sites would allow for a more detailed investigation of the associations between exposure to PACs and epigenetic changes and help isolate the impacts of PACs from other factors. Furthermore, it remains unclear whether shared DMRs associated with different sites have a functional role in gene expression responses. Further studies leveraging both transcriptomic and methylome analyses will be important for uncovering these relationships.

Functional impacts associated with PACs exposure have been observed in previous studies in humans (Ünlü Endirlik et al., 2023), laboratory animals (Billiard et al., 2002), and wildlife species (Willett et al., 1997; Woo, 2022), including birds (Perez-Umphrey et al., 2018). For example, a study conducted on sanderlings (Bianchini et al., 2021) that were dosed with a PACs revealed a decrease in the expression of liver basic fatty acid binding protein 1 (Lbfabp) and hepatic lipase (Lipc), suggesting that exposure to PACs could hinder the uptake, transportation, and metabolism of fatty acids, potentially leading to delayed migration departure timing (Bianchini and Morrissey, 2018), which is vital for long-distance migratory birds. Similarly, studies have shown that exposure to PACs can lead to the induction of specific genes, such as CYP1A (nestling herring gull, Lee et al., 1985; chicken embryo, Lee et al., 1986; herring gull, Peakall et al., 1989), which

plays an important role in PACs metabolism, and serves as a recognized and widely used measure of both PACs exposure and the molecular effects of PACs (Jönsson et al., 2011; Lara-Jacobo et al., 2019). Importantly, methylation changes in the CYP1A promoter region have been shown to be correlated with increased levels of CYP1A expression in developing chicken embryos exposed to PACs (Brandenburg and Head, 2018). Greater understanding of the functional links between methylation changes and the expression levels of PAC-associated genes is an important issue for further study.

The timescale of epigenetic responses to environmental perturbations in natural populations is poorly understood (Angers et al., 2020; Tian and Marsit, 2018). In this study, I analyzed two sites where wild black guillemots have experienced pollution from similar LMW PACs types, but with different exposure histories. At Postville, the population experienced an acute exposure of 3000L of crude oil spilled into the water. In contrast, at Nain, shipping traffic has been steadily increasing over the last 30 years with the opening of new shipping lanes (Oceans North Canada, 2016; Pizzolato et al., 2013). While the general methylation difference relative to the reference site was remarkably similar between these two sites (95% and 94% of DMRs showing hypermethylation, respectively), I found 1.7 times as many DMRs at Postville as at Nain (382 vs. 223). This suggests that the acute PACs exposure caused by the oil spill might have led to a more widespread genomic response than that occurring at Nain, where PACs exposure has been lower and more gradual. Future temporal sampling will help to establish whether the broad genomic response I have observed at Postville immediately following the oil spill might diminish to match Nain more closely, or if perhaps the number of DMRs at Nain will rise with further increases to shipping traffic and concomitant exposure to elevated PACs levels.

In this study, I explored the link between environmental oil exposure and methylation patterns in wild seabirds. I identify a core set of DMRs associated with oil exposure in black guillemot. In addition, I found evidence that varying composition of oil contaminants and length of exposure are associated with differences in methylation patterns. Without an annotated reference genome, it is difficult to identify the potential functional role of loci involved in these methylation differences. Obtaining a reference genome would allow for functional analysis in future studies that could provide insights into the mechanisms underlying the observed epigenetic responses and help to disentangle the effects of oil pollution and other environmental stressors. Nonetheless, the loci identified here provide good candidates for further research investigating the functional role of DNA methylation in response to anthropogenic oil pollution.

Supplemental material



Figure S1. Mean and ± SD of DNA methylation level of each methylation type (CG, CHG and CHH) at each study site.



Figure S2. Proportion of each methylation type contributing to the total amount of methylated cytosines.



Figure S3. Principal Components Analysis in REFERENCE-SPILL comparison of (A) all CpG loci with 5x coverage across samples and (B) DMRs. Methylation status of individual CpG loci explained 15.94% of variation between samples when considering all CpG loci. Methylation status of DMRs explained 49.77% of sample variation.



B

A

Figure S4. Principal Components Analysis in REFERENCE-SHIP comparison of (A) all CpG loci with 5x coverage across samples and (B) DMRs. Methylation status of individual CpG loci explained 14.76% of variation between samples when considering all CpG loci. Methylation status of DMRs explained 55.95% of sample variation.



Figure S5. Principal Components Analysis in REFERENCE-SEEP comparison of (A) all CpG loci with 5x coverage across samples and (B) DMRs. Methylation status of individual CpG loci explained 13.9% of variation between samples when considering all CpG loci. Methylation status of DMRs explained 53.03% of sample variation.



Figure S6. Principal Components Analysis in REFERENCE-SHIP-SPILL-SEEP comparison of (A) all CpG loci with 5x coverage across samples and (B) DMRs. Methylation status of individual CpG loci explained 13.9% of variation between samples when considering all CpG loci. Methylation status of DMRs explained 16.97% of sample variation.

CpG methylation clustering



Figure S7. Dendrogram showing hierarchical clustering of methylation levels of all CpG loci from all four sites. REFERENCE are shown in red, SPILL are shown in green, SHIP are shown in yellow, and SEEP are shown in blue.

Data accessibility

Raw Illumina sequence reads, and all analysis script will be shared once permission is granted from ECCC.

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